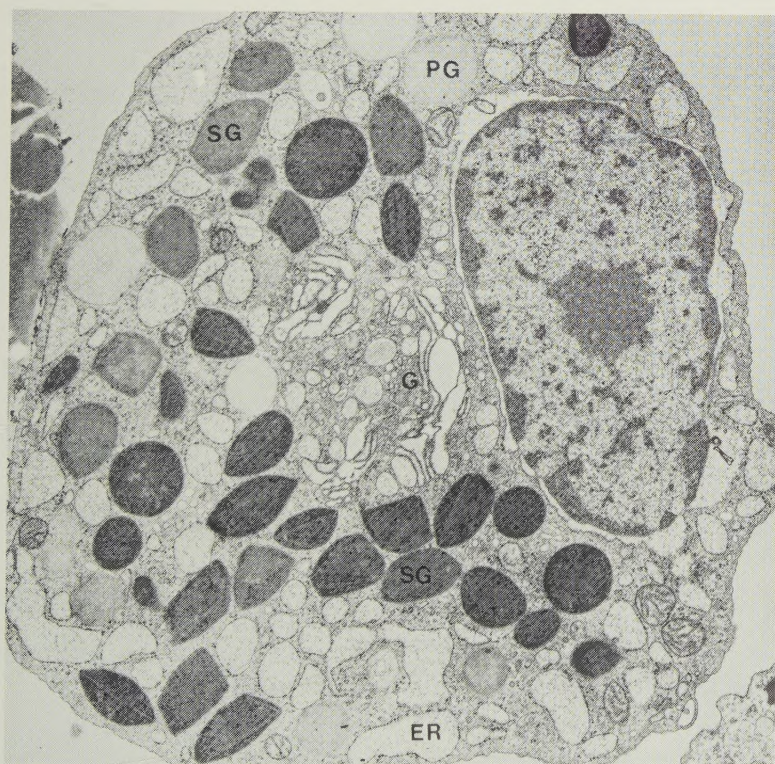


ON THE FUNCTION OF EOSINOPHIL GRANULOCYTES

— with special reference to the eosinophil
cationic protein

Ingemar Winqvist



Lund 1985

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Title and subtitle ON THE FUNCTION OF EOSINOPHIL GRANULOCYTES - with special reference to the eosinophil cationic protein			
Abstract The eosinophil cationic protein (ECP) was used as eosinophil marker in studies of eosinophil degranulation in vivo and in vitro and for characterization of eosinophils in eosinophilia. Challenge in patients with food intolerance demonstrated that eosinophils participate in allergic reactions and release ECP. In vitro release of ECP through reversed endocytosis occurred after adherence of eosinophils to a complement-coated surface. Cytochalasin B, trypsin, hydrocortisone, concanavalin A and Zn^{2+} reduced the release of ECP. The findings from modulation of ECP release suggest differences in mechanisms between degranulation in eosinophils and neutrophils. Eosinophils adhered to an antibody-coated surface by several mechanisms. Some Fc receptor dependency was demonstrated but nonspecific adherence seemed to be more important. In idiopathic hypereosinophilia a population of eosinophils of abnormally low density was detected. These eosinophils had low ECP content, increased expression of surface receptors and increased oxygen consumption suggesting that they were activated and degranulated in the circulation. ECP released from such eosinophils may be responsible for the tissue damage in hypereosinophilia. Biochemical characterization of purified ECP revealed several peptides with almost identical amino acid composition. The amino-terminal sequence was determined for one peptide. No homology was found with polypeptides of known amino acid sequence. Biosynthetic labeling demonstrated biosynthesis of ECP with molecular weight of 22,000 which was processed to lower molecular weight ECP corresponding to granule-bound ECP.			
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University of Lund, Sweden

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This thesis is based on the following papers, which will be referred to by their roman numerals.

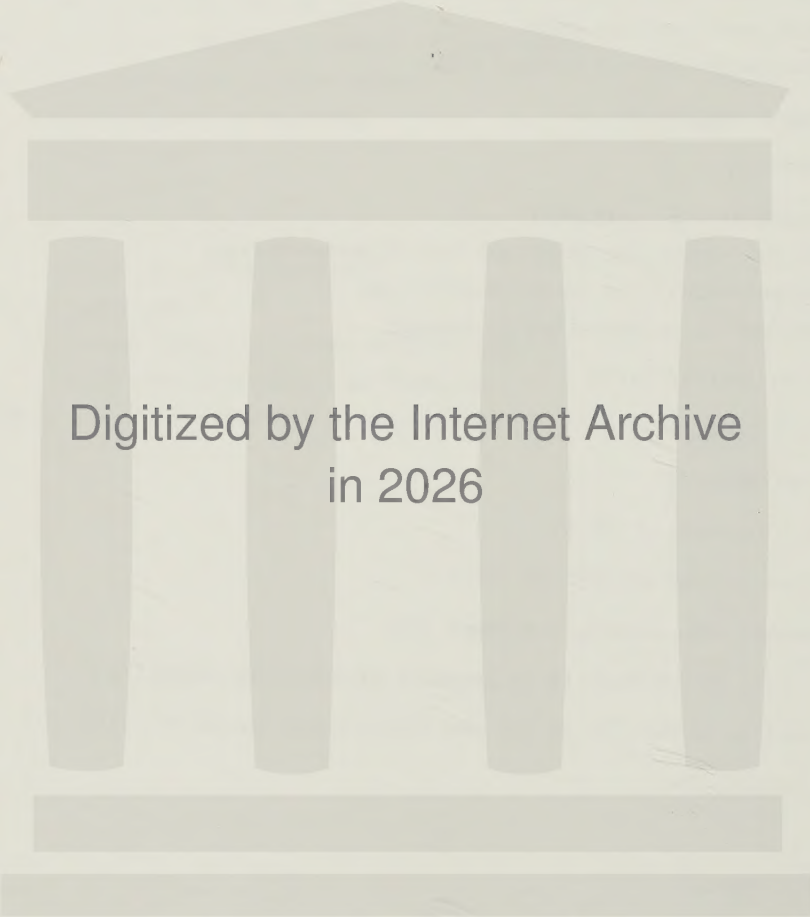
- I. Winqvist I, Olsson I, Werner S, Stenstam M.
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Allergy 36: 419-423, 1981.
- II. Winqvist I, Olofsson T, Olsson I.
Mechanisms for eosinophil degranulation; release of the eosinophil cationic protein.
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- V. Winqvist I, Olofsson T, Olsson I, Persson AM, Hallberg T.
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- VI. Olsson I, Persson AM, Winqvist I.
Biochemical properties of the eosinophil cationic protein (ECP) and studies of its biosynthesis in vitro in marrow cells from patients with eosinophilia.
Submitted for publication.

ABBREVIATIONS

EPO	eosinophil peroxidase
SRS-A	slow reacting substance of anaphylaxis
EDN	eosinophil-derived neurotoxin
PAF	platelet-activating factor
MBP	major basic protein
ECP	eosinophil cationic protein
ECF-A	eosinophil chemotactic factor of anaphylaxis
HETE's	hydroxyeicosatetraenoic acids
HES	hypereosinophilic syndrome
LTs	leukotriens
LTC ₄	leukotrien C ₄
Con A	Concanavalin A
PMA TM	phorbol myristate acetate
Cyto B	Cytochalasin B
EA	antibody-sensitized sheep erythrocytes
EAC	complement-coated sheep erythrocytes
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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INTRODUCTION

The eosinophil granulocyte was recognized in 1879 by Paul Ehrlich, who noticed the characteristic staining of its cytoplasmic granules with the acid dye eosin (1). An increased number of eosinophils (eosinophilia) is found in blood and affected tissues of patients with allergic and parasitic diseases and during the course of certain neoplastic, inflammatory and immunodeficiency diseases. Despite numerous investigations the function of the eosinophil is still mysterious. The presence in the cell of agents with potent parasitocidal activities suggests a role in defense against helminthic infections. The eosinophil has also been suggested to participate in acute inflammatory processes by neutralizing humoral mediator systems. On the other hand eosinophilia is sometimes associated with tissue damage. Thus the cytotoxic power of the eosinophil can be directed towards the host itself. The eosinophil cationic granule proteins are of special importance because they show damaging effects on several mammalian cells.

PREVIOUS INVESTIGATIONS

Eosinophil morphology

The eosinophil, like the neutrophil, contains two types of granules. One type is large granules with an angular core or crystalloid structure surrounded by a matrix (2). These granules show affinity for eosin. Another type is smaller cytoplasmic granules rich in acid phosphatase and arylsulfatase (3). In addition mature eosinophils contain organelles with unknown content called specific microgranules (4) with a ring or dumb-bell shaped structure; these granules are more numerous during eosinophilia (5).

Surface receptors

Eosinophils have membrane receptors for the Fc part of IgG and for the complement components C3b, C4 and C3d (6). The percentage of eosinophils forming rosettes with IgG coated erythrocytes varies depending on the species in which the IgG antibody is produced (7, 8). It has not been settled if eosinophils from patients with parasitic infections and hypereosinophilia express more receptors for IgG than eosinophils from healthy individuals (8, 9, 10). Human blood eosinophils also have a receptor for the Fc part of IgE (11, 12, 13). Eosinophilotactic mast cell derived substances can obviously increase the percentage of eosinophils with receptors for complement but not receptors for the Fc part of IgG (14, 15).

Granule composition and function of granule constituents

The major eosinophil granule proteins and suggested functions are summarized in Table 1.

The eosinophil peroxidase (EPO) exerts in the presence of hydrogen peroxide and a halide ion a cytotoxic effect against tumor cells (16) fungi (17), bacteria (18, 19) and parasites (20). It can also initiate secretion of histamine from mast cells (21).

Arylsulfatase has been reported to inactivate the smooth muscle contractile activity of slow-reacting substance of anaphylaxis (SRS-A) (22). This observation has, however, been refuted because purified eosinophil arylsulfatase does not inactivate the leukotriens that SRS-A consists of (23). EPO, however, can inactivate these leukotriens (24).

The eosinophil-derived neurotoxin (EDN) produces Purkinje cell injury and demyelination in the brain (25, 26).

Phospholipase D inactivates the platelet-activating factor, PAF (27).

Table 1. Eosinophil granule contents

Eosinophil peroxidase (EPO)	Peroxidative killing Histamine release
Arylsulfatase	-
Eosinophil-derived neurotoxin (EDN)	Cytotoxic
Phospholipase D	Inactivates PAF
Lysophospholipase D	Charcot-Leyden crystals
Major basic protein (MBP)	Cytotoxic to cells and parasites
Eosinophil cationic protein (ECP)	Cytotoxic to cells and parasites Activates coagulation

Lysophospholipase - the Charcot-Leyden crystal protein

One constituent of the eosinophil, associated with membranes, is lysophospholipase. It is the sole protein constituent of the Charcot-Leyden crystals (28, 29) which are present in tissues with eosinophil infiltration. Lysophospholipase may protect the eosinophil against cytotoxic effects of lysophospholipids (28). This enzyme is also present in human basophils (30).

Major Basic Protein (MBP)

Early studies suggested the presence of arginine-rich proteins in eosinophils (31). Gleich et al. isolated a Major Basic Protein (MBP) of eosinophil granules from guinea pigs (32). The MBP is a strongly basic polypeptide with a molecular weight of 9,300 in the human (33) and 11,000 in the guinea pig (34). It was reported that MBP constitutes about 50 % of the total granule protein of the eosinophil and that the crystalline core of eosinophil granules consists of MBP (35). MBP is detectable also in basophils (36). MBP and the Charcot-Leyden crystal protein are separate proteins (33).

The MBP damages schistosomules of *Schistosoma mansoni* (37) and newborn larvae of *Trichinella spiralis* (38). In addition it is toxic to several mammalian tissue cells including tracheal epithelium (39, 40). MBP is probably released into respiratory tissues in severe asthma and it has been identified in lung tissues of patients with bronchial asthma (41). Increased MBP is found in sputa of asthmatic patients at levels associated with damage to tracheal epithelium (42). Thus MBP may have a damaging effect in asthma especially as it also induces histamine release from human basophils (43).

Eosinophil cationic protein (ECP)

Olsson et al. identified in human eosinophils arginine-rich proteins (44, 45) designated Eosinophil Cationic Proteins (ECP). ECP is a zinc-containing single-chained protein with a molecular weight of 21,000. The isoelectric point is higher than pH 11. ECP contains 18 different amino acids, including 15 prolyl residues, one lysyl residue, two residues of tryptophan, an aspartate to glutamate ratio of 3:1, 21 residues of arginine, ten half-cystine residues and one methionyl residue (44). It does not have any bactericidal or esterolytic activity and it does not inhibit guinea pig anaphylatoxin or histamine-induced contraction (45). ECP neutralizes heparin (46) and both coagulation (47) and fibrinolysis (48) are affected. Thus it shortens the plasma coagulation time by activation of the Hageman factor and it activates fibrinolysis by enhancement of the urokinase induced plasminogen activation.

ECP kills schistosomules of *Schistosoma mansoni* at concentrations of less than 10^{-7} M (49). When injected into brain ventricles of guinea pigs, ECP damaged the brain tissue especially Purkinje cells of cerebellum and the pyramidal cells of Hippocampus (50). The effect of ECP on the Purkinje cells resembles the damage in the so-called Gordon phenomenon which was originally produced by injection into the cerebrospinal fluid of lymph node extracts from patients with Hodgkin's disease (51). ECP also shows cytotoxic effect on isolated heart muscle cells in vitro (52, 53).

Sensitive radioimmunoassays have been developed for both MBP and ECP (54, 55, 56) which allow measurements in serum. The ECP concentration in normal serum is in the range from 5 to 55 $\mu\text{g/l}$ (56) and the MBP level is in the range of 320-800 $\mu\text{g/l}$ (55). During acute inflammatory reactions eosinophils often disappear from the blood probably attracted to the inflammatory process (57, 58, 59). However, serum ECP levels are often raised in acute inflammatory diseases (60, 61, 62) suggesting that the eosinophils may participate in such diseases. MBP, ECP and EDN are distinctive cationic proteins of the human eosinophil as judged by immunochemical and physicochemical properties (63).

Eosinophil degranulation

Eosinophils phagocytose less efficiently than neutrophils (64, 65). Therefore degranulation to the exterior may be more common than degranulation into a phagocytic vacuole. Adherence to a surface is usually a prerequisite for degranulation. Eosinophils adhere to schistosomules in the presence of antibodies much better than neutrophils despite the fact that neutrophils have more and stronger Fc receptors for bound IgG than eosinophils. This may be explained by irreversible binding by eosinophils to the parasite as a result of degranulation and perhaps release of cationic proteins (66, 67).

The mechanisms for degranulation are not clarified in any detail. Release of arylsulfatase has been shown after binding of eosinophils to a surface through receptors for complement (68). ECF-A alone initiated release of some arylsulfatase without requirement of a surface for adherence (69).

Several eosinophil enzymes are released after phagocytosis of opsonized zymosan (70). Non-phagocytosable antigen-antibody precipitates can induce extracellular release of granule contents (71). IgG-coated parasites can provoke secretion of basic proteins and peroxidase (72). Ingestion of immune complexes especially human IgE-rabbit-anti-IgE complex gave rise to secretion of peroxidase (73).

Eosinophilia

Eosinophilia occurs in atopic disorders and metazoan infections, where there is an interaction between antigen, IgE antibody and mast cells. However, a causal relationship is not found between levels of IgE and eosinophils. An increased production of eosinophils requires T lymphocyte products (74, 75, 76).

Cells from patients with eosinophilia are often used in studies of eosinophil function. These eosinophils may, however, show signs of degranulation, vacuolization and hypersegmentation suggesting that they have been "activated" or stimulated in vivo. In some studies eosinophils from patients with eosinophilia show increased receptors for IgG (9, 10, 77) and C3b (9) but not in another study (8). Such eosinophils are more potent than eosinophils from healthy individuals in the killing of antibody-coated schistosomules (78) and they show changes in metabolism and surface charge (79). The production of superoxide has been reported to vary among eosinophils from healthy individuals and patients with eosinophilia (80).

FUNCTIONS OF THE EOSINOPHIL

The eosinophil is supposed to play a role in immediate type hypersensitivity reactions and helminthic infection because these conditions are characterized by eosinophilia.

The eosinophil and immediate type hypersensitivity

The immediate-type hypersensitivity reaction is produced by the interaction of antigen with specific IgE on the surface of the mast cell resulting in degranulation. The biological effects of mast cells are results of the release of substances, which increase venular permeability, contract smooth muscles, influence the motility of leukocytes, affect the generation and release of biologically active material from other cell types and degrade substrates such as proteoglycans and collagen. The mast-cell mediated reaction e.g. in urticaria-angioedema is attributed primarily to the release of histamine, although other agents such as platelet-activating factor and SRS-A also play a role.

Eosinophils accumulate in response to the eosinophil chemoattractants histamine and ECF-A. Histamine gives, however, rise to eosinophil recruitment only in atopics and not in healthy individuals (81). ECF-A is secreted by IgE-sensitized mast cells or basophils after exposure to a specific allergen (82). Both ECF-A and histamine enhance eosinophil complement receptors (14, 15).

A role has been suggested for the eosinophil in modulating mast-cell dependent reactions (83). Certain enzymes of the eosinophil have the ability to degrade or inactivate products that are released by mast cells. The eosinophil peroxidase inactivates the leukotriens of which SRS-A consists (24). Phospholipase D destroys platelet-activating factor (27). Histaminase is supposed to inactivate histamine (84). MBP neutralizes heparin released from mast cell granules (33). Eosinophils also contain a so called eosinophil-derived inhibitor which inhibits degranulation of basophils (13, 85). Eosinophils phagocytose mast cell granules (86), leading to inactivation of the content of such granules. Thus the eosinophil seems to be able to neutralize some mast-cell products.

The suggested dampening role for the eosinophil in the immediate type hypersensitivity reaction is, however, based principally on in vitro findings and there are several objections against such a role in vivo. For instance the MBP initiates histamine release from basophils (43). Therefore MBP could actually augment the basophil degranulation instead of inhibiting it. Also the eosinophil peroxidase can degranulate mast cells (21). Some data suggest that the histamine release is even reduced in the absence of eosinophils (87). Therefore at the present time an unequivocal role of the eosinophil as a dampening factor in the immediate type hypersensitivity is not supported. On the contrary, it is possible that the eosinophil may have a cytotoxic effect in hypersensitivity reactions such as asthma and cause tissue damage.

The eosinophil and parasite infection

Infusion of an antieosinophil serum leads to a depletion of eosinophils with abolished resistance to *Schistosoma mansoni* (88) and enhanced susceptibility to *T. spiralis* infection in mice (89). These data indicate a role in vivo for the eosinophil in parasite infections.

The cytotoxic effect of the eosinophils for non-phagocytosable parasites requires direct contact. Eosinophils are supposed to attach to the parasite after complement or antibody dependent adherence leading to degranulation. After initial attachment, the binding of the eosinophil to the parasite becomes firmer in association with deposition of granule contents onto the surface of the parasite (66). The tegument of schistosomules can activate complement via the alternative pathway and parasites coated with C3 are more susceptible to killing by rat eosinophils than antibody coated organisms (90, 91). Killing of schistosomules by human eosinophils is demonstrable only in the presence of complement (92).

The enhancement of eosinophil complement receptor activity by ECF-A, histamine and HETE's (14, 15, 93) may lead to an increased susceptibility of complement coated parasites to eosinophil-mediated damage. Blood eosinophil cytotoxicity is also enhanced by growth factors for eosinophil progenitors, so called colony-stimulating factors (94) and certain lymphokines (95). Eosinophils from patients with eosinophilia have an enhanced helminthotoxic capacity (78).

Parasites may be killed in several ways by eosinophils. Phospholipases may initiate damage to lipid-rich surfaces of parasites. Eosinophil peroxidase exerts in the presence of H_2O_2 and a halide ion a cytotoxic effect on schistosomules (20). Both MBP and ECP kill schistosomules (37, 49). The relative importance of different parasitocidal agents of the eosinophil is, however, not established and the agents mentioned may act in concert.

Eosinophils as mediators of disease

The occurrence of organ involvement that may accompany eosinophilia suggests that the eosinophil can damage the host and that studies on the role of eosinophils in HES may be of key importance to understand biological effects of eosinophils.

HES is characterized by blood and bone marrow eosinophilia with tissue infiltration by eosinophils and multi-organ dysfunction. The organ most commonly involved is the heart, which is usually affected by an endomyocardial fibrosis. The eosinophils in HES have an increased capacity to bind complexed IgG (7) and an increased capacity to kill antibody coated liver cells and human heart cells (77); increased cytotoxicity may be related to an altered metabolic state including an increased capacity to generate superoxide radicals (80). Many blood eosinophils are degranulated in HES and raised serum levels of ECP (96) MBP (55) and the Charcot-Leyden crystal protein (97) have been found. Extracellular ECP may therefore contribute to the development of cardiac injury and myocardial disease in patients where serum levels are high (52). It is also possible that myocardial disease occurs because of local infiltration of eosinophils.

The effects of eosinophil basic proteins on cultured intestinal, splenic, cutaneous and peripheral blood mononuclear cells have been examined. MBP damages all these cells (39). As mentioned above MBP also has a cytotoxic effect on tracheal epithelium and is therefore likely to contribute to the cellular injury that occurs in asthma. Eosinophils may produce leukotriens (LTs) including preferentially LTC_4 from the lipoxygenase pathway (98). The

smooth muscle constricting and bronchospastic activity in vivo of LTC_4 (99, 100) may have a causative role in asthma and in other conditions with eosinophilia.

The results from immunochemical studies using monoclonal antibodies to ECP (101) showed large amounts of ECP in the granulomas of biopsy specimens and tissues obtained at necropsy from patients with the Churg-Strauss syndrome (allergic granulomatosis and angiitis). Many degranulating eosinophils were seen to be migrating into the granulomas (101). The presence of large amounts of eosinophil cationic proteins within the granuloma may suggest that eosinophils play a central role in the development of lesions in the heart and other tissues of this disease.

THE PRESENT INVESTIGATION

Aims of the investigation

ECP is a specific marker for eosinophils. Detection of ECP can therefore be used to investigate phenomena related to degranulation in eosinophils. This possibility has been taken advantage of in the present dissertation where the aim has been to study functions of the eosinophil related to the degranulation process. It was thought that the questions asked might shed light on the role of eosinophils in health as well in diseases characterized by eosinophilia.

The following questions were asked:

1. Is ECP released in vivo in patients with allergic disease? An extension was performed of earlier work and serum-ECP was measured during challenge in patients with food intolerance and allergic rhinitis.
2. What are the mechanisms which govern degranulation in eosinophils and the release of ECP? An in vitro system was designed to induce degranulation and this process was modulated in a number of ways to define mechanisms involved. The effect of zinc and other cations on ECP release was studied in a separate investigation.
3. By which mechanisms do eosinophils adhere to a surface? Prerequisite for degranulation is attachment of the cells to a surface. Therefore adherence of eosinophils was investigated by use of Sepharose or Sephadex particles; the dependency on the receptor for Fc(IgG) was studied.
4. Do eosinophils from patients with eosinophilia sometimes show signs of degranulation and other signs of "activation", which may be of importance to explain damage to tissues in such patients?
5. What are the biochemical characteristics of ECP and is it possible to study its biosynthesis and storage in granules using biosynthetic labelling of marrow cells from patients with eosinophilia?

METHODS AND RESULTS

In vivo release of ECP (I)

In order to investigate the in vivo release of ECP, patients with food intolerance and allergic rhinitis were studied. In 18 patients with a history indicating intolerance against cow's milk and in three controls a challenge with cow's milk was performed. The number of eosinophils and the serum-ECP were measured 15, 30, 60 min, 2, 4, 6 and 8 h after challenge.

6/18 patients with milk intolerance and the three healthy individuals tested showed neither changes of serum ECP or eosinophil counts nor any symptoms.

In 5/18 patients with food intolerance the challenge produced a decrease in the eosinophil count but serum-ECP showed peak-like increases within 2 h followed by a decrease. These patients showed late symptoms often from extraintestinal organs e.g. as arthralgia.

7/18 patients with food intolerance showed a decrease in serum-ECP with the lowest levels after 2 h and to some extent also a decrease in eosinophil count. All these patients showed early gastrointestinal symptoms.

The decrease of serum-ECP most likely depends on increased elimination and perhaps consumption in the target organ. The decrease in blood eosinophils occurring simultaneously may also be partly responsible for a decreased serum-ECP.

Five patients with seasonal allergic rhinitis due to grass pollen were challenged by application of a pollen extract in the conjunctival sac. In spite of the fact that the target organ is small in this disease, four of five patients showed a more or less pronounced peak like increase of serum-ECP approximately 1 h after challenge again suggesting extracellular release of ECP from eosinophils. Pretreatment with disodium cromoglycate, which inhibits mast cell degranulation, abolished both the symptoms and the increase of serum-ECP upon challenge suggesting that the changes of serum ECP or release of ECP are somehow dependent on mast cell degranulation. However, an antihistamine drug did not abolish the increase of serum-ECP but perhaps delayed it. Thus the release of ECP in vivo is independent on histamine release from mast cells. These studies as well as earlier ones (62) confirm that ECP is released in vivo in allergic reactions.

In vitro release of ECP (II, III)

Degranulation and release of ECP was induced by exposure of purified eosinophils to a large surface consisting of Sephadex beads coated with serum. Approximately 15% of the cellular ECP was secreted both in eosinophils from patients with eosinophilia and healthy individuals. The ECP release was reduced when eosinophils were exposed to Sephadex beads pretreated with heat-inactivated serum indicating that such heat labile serum factors like complement components were of importance. Exposure of eosinophils to untreated Sephadex did not result in degranulation. Treatment of eosinophils with trypsin, which destroys C3 receptors (102), resulted in a decrease in ECP-release during incubation with serum-treated Sephadex, suggesting that interaction of eosinophils with serum-treated Sephadex through C3 receptors was a prerequisite for degranulation. This was established using serum from a patient with C3-deficiency. Thus Sephadex coated with this serum did not induce release of ECP from added eosinophils (Fig. 1). It is therefore concluded that eosinophils are attached to serum-treated Sephadex by their receptors for C3.

Soluble factors such as zymosan-treated serum (ZTS), ConA and PMA, which can provoke secretion from neutrophil specific granules (103), did not by themselves induce ECP release.

Both cytochalasin B, cytochalasin D and hydrocortisone inhibited the Sephadex-stimulated ECP-release. Adherence of eosinophils was not reduced by these agents; the inhibitory effect therefore seems to be a direct effect on the degranulation process.

For comparison, neutrophil release of myeloperoxidase and lactoferrin was measured upon exposure of neutrophils to serum-coated Sephadex beads. The relative release of myeloperoxidase and lactoferrin was lower than the relative ECP release and contrary to ECP release, it was not inhibited by cytochalasin B. These data suggest that mechanisms for degranulation are not identical in eosinophils and neutrophils.

No direct relationship was found between degranulation and stimulation of an oxidative burst in as much as some soluble mediators induced a high respiratory burst in eosinophils without concomitant ECP-release.

In order to further investigate the mechanism of degranulation in eosinophils and the difference in this respect between eosinophils and neutrophils, we have studied the effects of zinc, manganese, calcium, magnesium and lithium on the release of ECP induced after exposure of eosinophils to Sephadex beads coated with serum. Both calcium and magnesium are prerequisites for optimal release from eosinophils. The release of ECP was only slightly stimulated after addition of either of these cations alone suggesting an additive effect of calcium

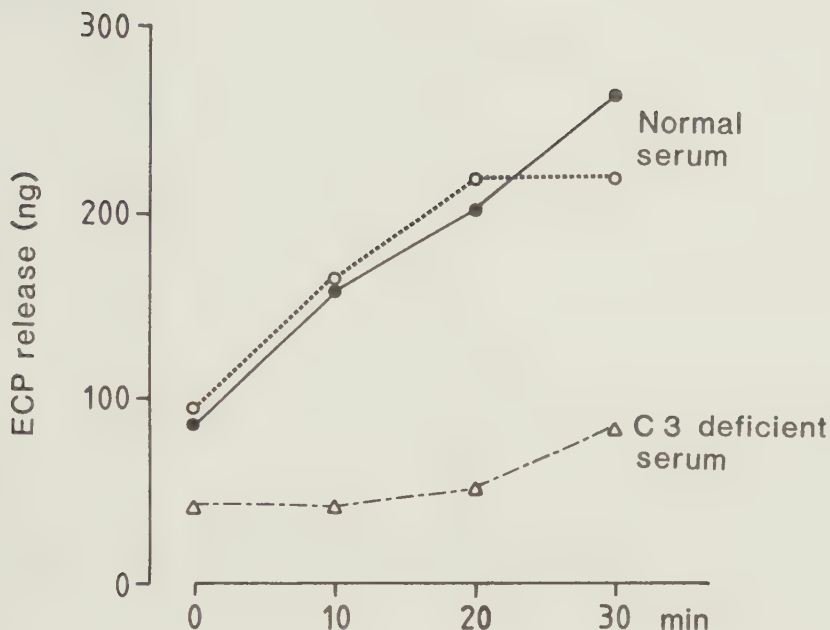


Fig. 1. Failure of Sephadex coated with C3 deficient serum to induce ECP-release

Serum from a patient with C3 deficiency (approximately 10% of normal level) was passed over a column of anti-C3 coupled to Sepharose to remove traces of C3 and was then dialyzed against Hanks' balanced salt solution before use; (this preparation was a generous gift from Dr. Ulf Johnson, Dept of Immunology, University of Lund). This C3 deficient serum was used to coat Sephadex beads which were then incubated with isolated normal eosinophils (Δ ----- Δ) as described in detail in paper II. Normal serum ($\bullet$ ----- $\bullet$) and normal serum which had been dialyzed against Hanks' balanced salt solution ($\circ$ ----- $\circ$) were run as controls.

and magnesium. ECP was, however, released even in the absence of these cations. The addition of zinc reduced the release in a dose-dependent fashion both in the presence and absence of calcium and magnesium. The inhibitory effect on ECP release by zinc seems to be due to an action on the cell membrane since the effect was immediate and reversible after washing of the cells even after prolonged preincubation with zinc. Manganese reduced the release of ECP only in the presence of calcium and magnesium suggesting a different mechanism for inhibition by manganese and zinc. Lithium like manganese reduced the release of ECP.

Eosinophil adherence to a surface (IV)

In order to investigate the dependency on the receptor for Fc (IgG), attachment to albumin-Sepharose coated with the IgG-antibody of antialbumin, $F(ab')_2$ fragments or serum was measured. Also adherence to Sephadex beads and albumin-coated microtitration plates was investigated. About half of the eosinophils adhered spontaneously to all three different surfaces when not coated with the antibody whereas only 25% of neutrophils and less than 10% of mononuclear cells adhered.

The adherence of eosinophils to albumin-Sepharose increased slightly after addition of the IgG-antibody but not after addition of $F(ab')_2$ fragments indicating that the Fc region is responsible for some increase in adherence. This was also supported by the finding that incubation of eosinophils with Fc-fragments abolished antibody-mediated adherence. The fraction of adherent cells did not change after incubation of the antibody coated albumin-Sepharose with normal fresh serum indicating that complement factors may be of minor importance for adherence to an antibody-coated surface.

When eosinophils, which did not adhere during a first incubation, were incubated a second time with antibody-coated albumin-Sepharose a large fraction still adhered. Fc receptor negative cells adhered almost as much as Fc receptor positive cells again indicating that the Fc receptor dependency is of minor importance for the total adherence of eosinophils to the particles used. Sephadex beads treated with fresh serum provided a more efficient surface for eosinophil adherence than albumin-Sepharose treated with serum. Adherence to Sephadex was not inhibited by cytochalasin B. The adherence to anti-albumin-coated albumin-Sepharose was, however, inhibited by cytochalasin B indicating separate mechanisms for adherence to serum-treated Sephadex and to antibody-coated albumin-Sepharose.

Trypsin treatment of the eosinophils, which increases EA binding while destroying EAC binding did not increase Fc receptor dependent adherence. These results, too, suggest that the Fc receptor dependency is not the most important factor for eosinophil adherence to antibody-coated albumin-Sepharose.

Activation of eosinophils in patients with hypereosinophilia (V)

In order to compare the eosinophils from normal individuals with those from patients with eosinophilia we performed studies on density distribution, oxygen consumption upon adherence to serum-coated Sephadex and expression of cell surface receptors for IgG and complement. Abnormal low density fractions of blood eosinophils were detected in eosinophil leukemia and in HES. Both "light" and "heavy" eosinophils were seen. Low density eosinophils of HES showed morphological signs of degranulation consonant with the finding of a low content of intracellular ECP. Such cells exhibited a higher oxygen consumption than eosinophils with normal density both at rest and upon adherence to serum-coated Sephadex. Low density eosinophils showed a higher number of cells with Fc(IgG) and complement receptors than high density eosinophils. Furthermore 50% of light eosinophils showed Fc(IgG) receptors and 50% showed complement receptors while heavy eosinophils displayed 34% and 14% Fc(IgG) receptors and complement receptors respectively. All these findings suggest that eosinophils from some patients with eosinophilia may be activated in the circulation.

In comparative studies between blood and pleural exudate eosinophils the latter showed low density and higher spontaneous oxygen consumption presumably representing tissue eosinophils.

The normal density found for eosinophils of patients with parasite infections, eosinophil gastroenteritis, primary hepatocellular carcinoma and lymphoma suggests that abnormal eosinophil degranulation in the circulation may not be a common finding in these disorders.

Biochemical properties of the eosinophil cationic protein (ECP) and biosynthesis in marrow cells (VI)

It seems likely that the eosinophil cationic proteins of eosinophil, after secretion, are at least in part responsible for cytotoxic effects of these cells. Therefore an extended purification and biochemical characterization of ECP was begun to get a better understanding of the mechanisms behind its cytotoxic effects and to get polypeptides suitable for amino acid sequencing. It is also possible that knowledge of mechanisms behind the biosynthesis, processing and storage in granules of ECP could contribute to define differen-

ces between storage and secreted forms of ECP. Therefore bone marrow cells from patients with eosinophilia were used for radiolabeling of ECP in eosinophil precursor cells. Actually it was recently reported that one monoclonal antibody recognized both storage and secreted forms of ECP whereas another antibody only bound to ECP during secretion (127). Thus it seems that the antigenicity of ECP could be altered during degranulation.

ECP was isolated by methods previously described using gel filtration followed by E-aminocaproic acid Sepharose chromatography (44, 45). The purified ECP showed two distinct components when analyzed by SDS-PAGE with molecular weights of 19,500 and 16,700. Both forms showed complete immunologic identity using an polyclonal antibody raised by immunization of rabbits. In the present studies we have, however, detected a considerable microheterogeneity of ECP by use of high resolution chromatography on a Mono S cation exchange column eluted with a sodium acetate gradient pH 5.0. By isocratic elution with 0.9 M sodium acetate 5 separate components were resolved. All these components reacted quantitatively in radial immunodiffusion using polyclonal anti-ECP. Molecular weights varied between 19,500 and 16,000. Amino acid analyses showed almost identical results for ECP from different peaks. The elution pattern on high resolution chromatography was reproducible as identical separation patterns were seen in separate experiments using cells from different patients with eosinophilia as starting material for purification of ECP. By rechromatography on a Mono S column homogeneous polypeptides were obtained.

One homogenous ECP polypeptide with a molecular weight of 16,700 was used for amino acid sequence analysis. The NH_2 terminus consisted of an arginine residue. The amino terminal sequence was (in the standard one-letter code) R-P-X-Z-F-T-R-A-Z-W-F-A-I-Z-H-I-S-L-N-P-R-R-C-T-I-A-M-R-A-I-N-N-Y-. A computer search using the sequence for the 33 NH_2 terminal amino acid residues revealed no significant homology with polypeptides of known amino acid sequence indicating the ECP is a unique protein.

For biosynthesis studies of ECP, marrow cells from patients with eosinophilia were incubated with (^{14}C) leucine followed by extraction and immunoprecipitation with anti-ECP. After SDS-PAGE fluorography was used for visualization of newly synthesized ECP. The fluorograms revealed biosynthesis of ECP polypeptides with molecular weights of 22,000. By use of pulse-chase labeling experiments it was shown that some of the newly synthesized ECP with time was converted into ECP with a molecular weight of approximately 19,000. The addition of Monensin, a carboxylic proton ionophore that exchanges preferentially Na^+ ions

for protons, prevented the conversion of Mw 22,000 ECP into lower molecular weight products. Our results from biosynthetic labeling indicate that ECP is subject to some alterations in size subsequent to its biosynthesis.

GENERAL DISCUSSION

This thesis has provided new information on the mechanisms which govern the degranulation process in eosinophils as well as direct evidence that degranulation of eosinophils occurs *in vivo*. Furthermore characterization of eosinophils in the HES indicated signs of "activation" and degranulation. The marker of eosinophils utilized in these studies was the unique eosinophil granule polypeptide ECP. New data were obtained on the biochemical characterization of ECP; a partial amino acid sequence was determined. It is not entirely sure that the release of ECP is an adequate measure for the discharge of all the different granule proteins in eosinophils; different localization of various granule proteins to the crystalloid or the matrix of the granule may influence their release. However, several reports indicate that ECP may have a number of biological effects e.g. on cells (50, 52) and on coagulation (47, 48). These effects may be of importance to explain the action of eosinophils in allergic diseases, parasite infections and in high eosinophilia states of unknown etiology. From this point of view too ECP seems to be a relevant marker for studies on eosinophil degranulation.

As the eosinophil has the highest density of all leukocytes most of the methods used for isolation of eosinophils are based on density gradient centrifugation. In paper II and V a method based on Percoll density gradient centrifugation was adopted (104). The osmolarity had to be carefully controlled as hyperosmolarity disturbed the separation by increasing the density of neutrophils. The recovery was rather low and the capacity to isolate eosinophils from healthy individuals was limited. Therefore we instead applied the method described by Vadas (105) using metrizamide gradients. This method gave high yields of 82-94 % pure eosinophils also from healthy individuals with normal eosinophil counts and the capacity was higher than for the Percoll method.

Degranulation is usually preceded by adherence of the cell to a surface. Therefore we also investigated the adherence of eosinophils to different surfaces and its dependency on serum factors and cell surface characteristics. Fig. 2 summarizes our findings on eosinophil adherence. Receptors for the Fc part of IgG and for complement are thought to play a role. Some Fc receptor dependent binding was shown to an antibody-coated surface (antialbumin-albumin-Sepharose). However, also Fc receptor negative cells adhered quite well to such a surface suggesting several different mechanisms for adherence to an IgG coated surface. It is even possible that Fc receptor dependency is not the most important factor for eosinophil adherence to an antibody-coated surface. Trypsin treatment of the eosinophils, which increases EA binding while destroy-

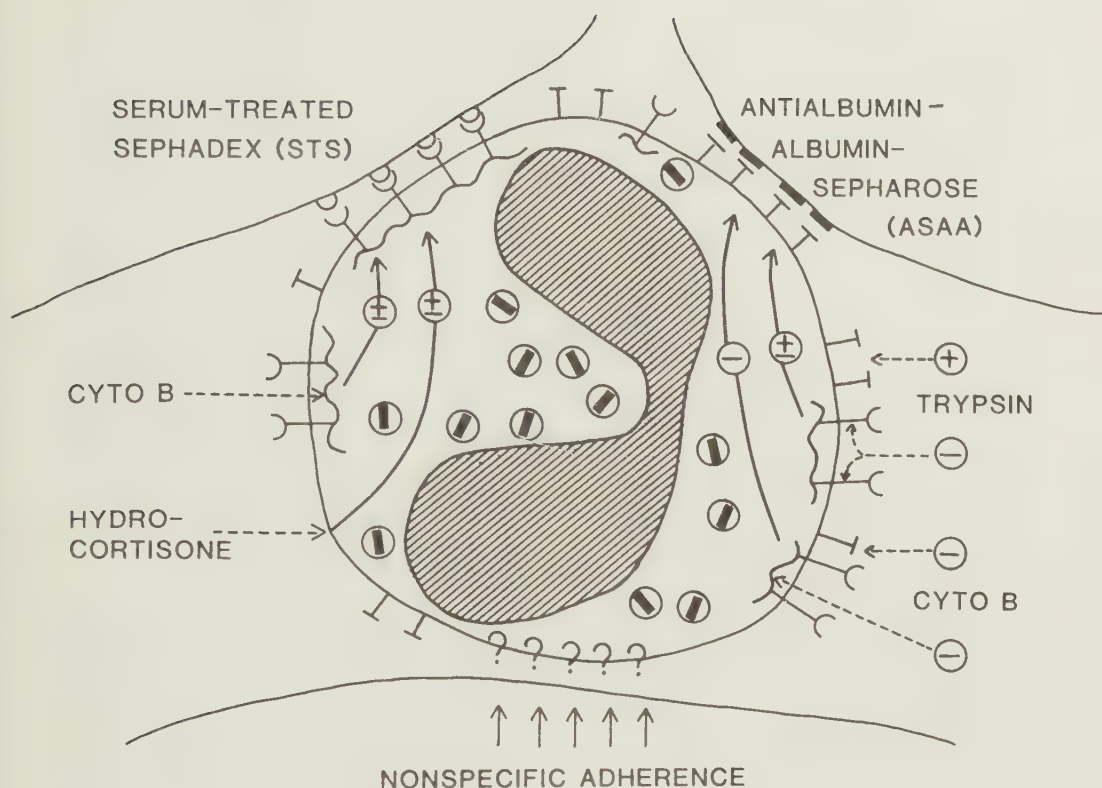


Fig 2. Adherence of eosinophils to serum-treated Sephadex (STS) and antialbumin-albumin-Sepharose (ASAA)

$\oplus$ = increase; $\ominus$ = reduction; $\oplus\ominus$ = no effect.

(STS). Cyto B, which destroys microfilaments (~~~~) and inhibits EA binding (T), and hydrocortisone did not affect the adherence to STS. (ASAA). Trypsin, which increases EA binding (T) while destroying EAC binding (Y), did not affect adherence to ASAA. Cyto B reduced the adherence to ASAA.

The mechanism of "nonspecific adherence" (bottom) is unknown.

ing EAC binding (102), did not increase Fc receptor dependent adherence, which also suggests that Fc receptor dependency is not the most important factor for eosinophil adherence. Furthermore Fc receptors are expressed to a much lower extent on eosinophils than on neutrophils, which also may indicate that they are of minor importance for eosinophil adherence. A high "nonspecific" binding seems to be a typical property of the eosinophils; neutrophils and mononuclear blood cells show much less "nonspecific" adherence.

Cytochalasins are known to inhibit the binding of eosinophils to IgG coated red cells (102) and cytochalasin B actually abolished some binding to the antibody-coated surface. It was however not possible to decide if it was Fc receptor mediated or nonspecific binding that was inhibited in this case. Cytochalasin B did not, however, inhibit the adherence of eosinophils to serum-treated Sephadex which presumably is mediated through complement receptors. Sephadex treated with fresh serum was actually a most efficient surface for eosinophil adherence suggesting that complement receptors mediate efficient binding of eosinophils to a surface.

Degranulation of neutrophils can occur in several ways such as through lysis, regurgitation during phagocytosis, reversed endocytosis and soluble factor-induced secretion (106). Of these, reversed endocytosis may be the most important for the eosinophil. Thus it has been reported that some arylsulfatase is released from eosinophils after adherence to serum-treated Sepharose (68), which represents reversed endocytosis. In this case the binding of eosinophils occurred through receptors for activated complement. Likewise serum-coated Sephadex, which was used in the present study provided an opsonized surface too large to ingest and induced extracellular secretion of ECP by reversed endocytosis. This model may therefore be relevant for the physiological action of the eosinophil e.g. on the surface of opsonized parasites where it degranulates and kills the parasite.

Fig. 3 summarizes our findings on eosinophil degranulation. After complement receptors were destroyed with trypsin (102), exposure of eosinophils to serum-treated Sephadex did not induce ECP release. Furthermore, Sephadex coated with complement-inactivated or C3-deficient serum did not induce ECP release. Soluble stimuli such as zymosan-treated serum, concanavalin A and PMA, which induce degranulation in neutrophils (103), were not able to induce release of ECP. Therefore adherence to a surface via C3 receptors seems to be of general importance for eosinophil degranulation.

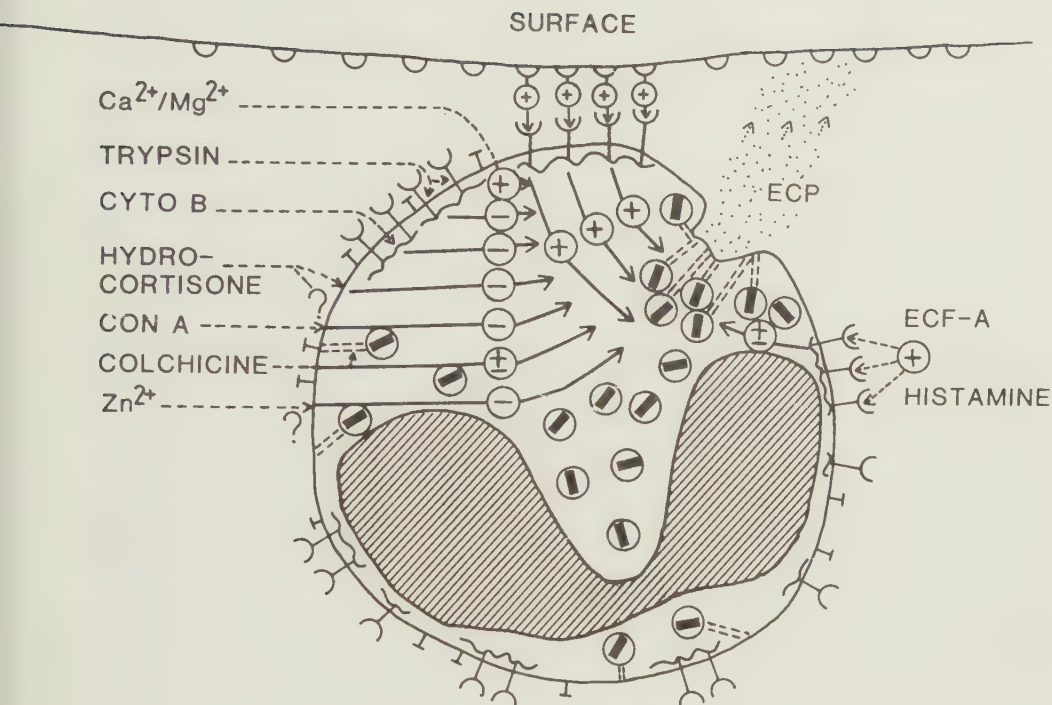


Fig.3. Release of ECP induced by complement-coated Sephadex (surface) and differences in mechanisms of degranulation between eosinophils and neutrophils (PMNs)

⊕ = increase; ⊖ = reduction; ⊕ = no effect.

$\text{Ca}^{2+}/\text{Mg}^{2+}$ are prerequisites for optimal release from both eosinophils and PMNs. Trypsin destroys complement receptors (Y) and reduces release of ECP. Cyto B depolymerizes microfilaments (~~~~) and reduces release from eosinophils. Hydrocortisone, which inhibits Fc receptor expression (T) in PMNs, reduces the release of ECP. Con A reduces release by an unknown mechanism. Colchicine, which destroys microtubuli (=====), did not affect the release of ECP. Zn²⁺ inhibits release of unspecific collagenase from PMNs and ECP from eosinophils.

ECF-A and histamine enhance complement receptors but did not affect the release of ECP.

Cytochalasin B, which depolymerizes the microfilaments (106), reduced the secretion of ECP without inhibiting the adherence of eosinophils to serum-treated Sephadex beads. Thus it inhibited directly the degranulation process without affecting the adherence of the cells. Also hydrocortisone reduced the ECP release without interfering with adherence of eosinophils (Fig. 2). The mechanism for the inhibitory effect of corticosteroids on ECP secretion could not be determined. In the neutrophil, corticosteroids inhibit Fc receptor expression (107) and lysosomal secretion (108). Others have found that whole blood eosinophil adherence was reduced after in vivo administration of corticosteroids (109, 110). Isolated eosinophils, however, showed normal adherence after in vivo administration and the adherence was not affected by addition in vitro of corticosteroids (109).

Concanavalin A, a lectin, reduced the Sephadex-induced release of ECP from eosinophils. It is known that Con A induces a reversible adherence of eosinophils to schistosomules but without evidence of damage. However, after addition of a calcium ionophore the adherence became irreversible and eosinophil proteins were secreted followed by damage to the parasite (66). Con A also gives rise to perinuclear vacuoles in eosinophils. The vacuoles contain granule constituents but these are not released to the exterior (111). Thus our own and other data indicate that Con A inhibits the degranulation without affecting adherence of eosinophils.

Our results suggest differences in mechanisms of degranulation between eosinophils and neutrophils. Thus contrary to the ECP release of eosinophils, the release of myeloperoxidase and lactoferrin from neutrophils was not affected by cytochalasin B. Actually the secretion of neutrophil granule constituents is markedly stimulated by cytochalasin B (106). This drug interferes with actin fibril integrity but also with hexose transport and may alter calcium fluxes (106). Con A and PMA, which provoke secretion from neutrophil specific granules, did not by themselves induce ECP release, which further indicates a difference between neutrophil and eosinophil secretion. Moreover colchicine, which destroys the microtubuli and inhibits release from neutrophils (106, 112) had no inhibitory effect on Sephadex-induced release of ECP.

Eosinophil degranulation was found to have the same requirements for Ca^{2+} and Mg^{2+} as neutrophils. Zn^{2+} selectively inhibits the release of unspecific collagenase from neutrophils whereas Mn^{2+} in addition inhibits the release of myeloperoxidase, chymotrypsin-like proteins and lysozyme but stimulates the release of lactoferrin (113). Zn^{2+} also inhibited the secretion of ECP from eosinophils. This inhibition was immediate and reversible after washing the

cells suggesting that the effect is localized to the plasma membrane. Zn^{2+} is known to inhibit the activity of several plasma membrane associated enzymes such as ATPase and phospholipase (114). That Zn^{2+} should interfere with calmodulin, which regulates the activity of several enzymes (115), is unlikely, because trifluoperazine, an inhibitor of calmodulin, did not inhibit secretion of ECP.

No direct relationship was found between degranulation and stimulation of an oxidative burst in eosinophils since some soluble factors such as zymosan-treated serum and PMA induced a high respiratory burst without a concomitant ECP release. Obviously these events are not coupled in eosinophils; they can occur independently in neutrophils too (103). Thus for instance soluble C5a stimulated neutrophils to generate superoxide without provoking enzyme release (103). However, contrary to findings with neutrophils, pretreatment of eosinophils with cytochalasin B decreased Sephadex-stimulated enhancement of the oxidative metabolism. It is emphasized that this effect is not caused by inhibition of eosinophil adherence since that process was unaffected by Cytochalasin B.

Results from assays of serum-ECP may throw light on the involvement of the eosinophil in hypersensitivity reactions. In healthy individuals serum-ECP correlates with the blood eosinophil count (56). It is not clear if increased serum-ECP found in some allergic disease (62) is the result of release from circulating eosinophils or eosinophils degranulating in the tissues. Circulating immune complexes could induce some degranulation as judged by the finding that ECP is released upon interaction between eosinophils and immune complexes (II). It is, however, more likely that ECP is released in the tissues upon exposure to surfaces coated with immunoglobulin and complement with the escape of some ECP into the circulation.

Other work has shown variations in serum-ECP in some allergic diseases (62). The present work demonstrated changes in serum-ECP and eosinophil counts during symptom-inducing challenge in food allergy. Data on serum-ECP are somewhat difficult to interpret for several reasons. The rate of turnover in the circulation of ECP is not known; indirect evidence suggests a very rapid turnover (I; 62). The serum-ECP may or may not derive from circulating eosinophils, which represent only a minor part of the total eosinophil pool. The binding proteins for ECP may vary during pathological conditions with consequences for the rate of elimination. Despite these difficulties significant patterns were established. Thus a decrease of serum-ECP was seen in patients

with early symptoms during challenge but not in patients with late symptoms. The early symptoms may depend on an immediate type hypersensitivity reaction. It is, however, possible that the early symptoms depend on some type of inborn error of reactivity (idiosyncrasy), as patients with symptoms from intrinsic asthma (aspirin idiosyncrasy) have very low levels of serum-ECP despite the fact that they have eosinophilia (116). Decreases of serum-ECP may, however, depend on an increased rate of elimination and perhaps consumption in the target organ. The decrease in blood eosinophils occurring simultaneously may be partly responsible for a decreased serum-ECP. Initial peak-like increases of serum-ECP upon challenge were seen only in a few patients with food intolerance and were associated with late symptoms. Rapid variations in serum-ECP have also been provoked by inhalation of specific allergen in asthma patients (62). Such rapid changes in serum-ECP must result from release from circulating eosinophils or eosinophils activated in the lung.

Thus a general conclusion is that ECP is released in vivo e.g. during allergic reactions. The increase of serum-ECP seemed to be dependent on mast cell degranulation judging from inhibitory effects by disodium cromoglycate (I). Most likely mast cells are responsible for attraction of eosinophils to the site of the allergic reaction with subsequent degranulation as a result.

Also in the HES there is evidence of in vivo degranulation with raised levels of serum-ECP (53, 96); in this disease degranulated eosinophils are actually observed in the circulation (53, 96). The low density of some eosinophils, which we found in HES, can probably be explained by a partial degranulation in the circulation with release of granule contents, which could lead to toxic manifestations. Alternatively (although unlikely) low density cells of HES may represent an abnormal (preleukemic) population which is produced in the marrow with a low granule content. However, the correlation reported earlier between the number of morphologically appearing degranulated blood eosinophils and the serum level of ECP indicates that ECP is released from circulating eosinophils in patients with HES (53, 96).

There are a number of observations indicating toxic effects of ECP; the damage to the heart in the HES may result from such an effect on heart muscle cells (52, 53). Neurotoxic effects of ECP have also been described (117) and ECP may therefore be responsible for the central nervous system lesions which are the second most clinically important complication in the HES (118). Raised levels of ECP in the cerebrospinal fluid have actually been observed in patients with various neurological disorders without blood eosinophilia (119). Many of the

central nervous system lesions in the HES may, however, also be due to an embolus originating in the heart (96, 118). Thromboembolic events are rather common complications in the HES and ECP may be responsible as it affects coagulation as described above. Our findings in the HES of low density cells with increased spontaneous oxygen consumption and respiratory burst upon adherence to a surface may support the notion that the production of oxygen radicals as well could augment the cytotoxic effects of eosinophils (120).

In vivo administration of corticosteroids to three patients with hypereosinophilia and elevated levels of serum-ECP reduced as expected the number of eosinophils in 4-6 hours. Concomitantly serum-ECP was reduced to normal levels within 4-6 hours (unpublished data). These findings support the notion that ECP may be responsible for some of the cytotoxic effects in hypereosinophilia as corticosteroids have a beneficial effect in some of the patients with hypereosinophilia.

The existence of "light" and "heavy" eosinophils in the HES has later been reported by others (121, 122, 123). Opposite to our own findings light or "hypodense" eosinophils were shown to have a reduced capacity for production of oxygen radicals (123). In patients with blood eosinophilia due to parasitic diseases, two eosinophil fractions were found which differed in cytotoxic effect and surface receptors (121); eosinophils with lower density expressed a reduced binding capacity for IgG. The difference in density between the two populations tested was, however, rather small as compared to the difference in density between "heavy" and light" eosinophils that we found in HES. This may explain why the eosinophils with lower density expressed a reduced binding capacity for IgG and C3, whereas our "light" eosinophils had an increased binding capacity for IgG and C3.

Light eosinophils of HES may represent a subpopulation of activated eosinophils with a prolonged half-life in the circulation. Actually the blood half-life of Chromium- or Indium-labeled eosinophils was sometimes prolonged in hypereosinophilia (124, 125). Our findings in such cells of an increased expression of cell surface receptors, increased spontaneous oxygen consumption and increased respiratory burst upon adherence to a complement-coated surface suggest that circulating eosinophils from some patients with eosinophilia may be activated. Furthermore our finding of exudate eosinophils with low density and higher spontaneous oxygen consumption than blood eosinophils suggests that eosinophils are normally activated in the tissues. Eosinophils from patients with eosinophilia have been reported by some to express more receptors for Fc (IgG) than

normal eosinophils (9, 10, 77), but others could not verify these findings (8). There may, however, be other differences which are related to cell surface charge, lysosomal activation and hexose transport (79).

The abnormally low density of eosinophils from patients with eosinophil leukemia indicating a low granule content or abnormal granule composition is most likely a result of the malignant character of such cells rather than being secondary to degranulation. Thus, in contrast to findings in HES, spontaneous and stimulated oxygen consumption were not elevated. Furthermore, the ECP content was extremely low in one patient with eosinophil leukemia without a clear relationship to density of the cells indicating abnormalities of granule composition.

To explore the mechanisms for the cytotoxic effects on cells and parasites of ECP biochemical characterization may be useful. The extended purification and characterization of ECP of the present work revealed some interesting features. A considerable molecular microheterogeneity of ECP was detected and different ECP species may show differences in biological actions. Several of these components, which differed only slightly in molecular weight were purified to homogeneity and the amino acid compositions were found to be almost identical. The cytotoxic power of ECP may result from its extreme basicity (with an isoelectric point above pH 11). However, the cytotoxicity is not likely to be totally unspecific since some cells are sensitive and others are resistant. Because of difficulties in iodination of ECP, it has not yet been possible to assay binding to various cells and determine if specific receptors are present. The cytotoxic effect obviously results in an increased membrane permeability (52) but the mechanism for this is unknown. With radioiodinated ECP available it will also be possible to determine if the ECP effect depends on its internalization. An enzymatic action with a substrate on the cell surface is another possible explanation for the cytotoxicity. If the amino acid sequence is known of a protein with an unknown function, comparisons with other proteins with known amino acid sequences may give useful information. The NH_2 -terminus sequence obtained for ECP was aligned with known protein sequences and no specific homologies were detected. Minor homologies found with the receptor-binding site of diphtheria toxin are probably without significance.

Information about structural modifications of ECP was obtained by studies on its biosynthesis in marrow cells from patients with eosinophilia. It was previously shown that another eosinophil granule constituent, EPO, was synthesized as a precursor polypeptide, which was processed by proteolytic cleavage

prior to or subsequent to storage in granules (126). Similarly biosynthetic labeling demonstrated biosynthesis of ECP with a molecular weight of 22,000, which was slowly processed to lower molecular weight ECP corresponding to granule-bound ECP. Moreover other work indicated that ECP is further modified during degranulation (127).

Complete structural definition of ECP will require the use of DNA hybridization probes. Because a partial amino acid sequence for ECP has been established in this work it is now possible to synthesize corresponding oligonucleotides. These can be used for screening of a cDNA-library constructed from mRNA isolated from marrow cells of patients with eosinophilia. Subsequent determination of the complete nucleotide sequence for ECP should extend the possibilities to define the function of ECP and the role of eosinophils.

SUMMARY

The eosinophil is involved in allergic and parasitic disease but its function(s) has not yet been clarified. One key may be the unique eosinophil granule polypeptide ECP with cytotoxic effects on cells and parasites. In the present work ECP has been used as an eosinophil marker in studies of eosinophil degranulation in vivo and in vitro and for characterization of eosinophils in eosinophilia. Also mechanisms of adherence to a surface were investigated. An extensive biochemical characterization of ECP was performed. New information on the eosinophil was obtained within the following areas:

When challenge was performed in patients with food intolerance typical variations of serum-ECP were documented. Patients who responded with late symptoms showed peak-like increases of serum-ECP while those with early symptoms demonstrated decreased levels of serum-ECP suggesting increased rates of elimination or consumption in the target organ. These results demonstrate that eosinophils can participate in allergic reactions with degranulation and release of ECP.

An in vitro model consisting of Sephadex beads coated with serum presumably leading to complement activation was used for studies of degranulation in vitro. Eosinophils adhered via their C3-receptors to the beads resulting in release of ECP through reversed endocytosis. Corticosteroids, which rapidly reduce the serum levels of ECP in some patients with hypereosinophilia, inhibited release of ECP in vitro. Both cytochalasins and concanavalin A reduced the ECP release without affecting the adherence of eosinophils to the Sephadex surface. In addition Zn^{2+} inhibited the secretion of ECP probably by an effect on the plasma membrane. These findings from modulation of ECP release suggest differences in mechanisms between degranulation in eosinophils and neutrophils. The model used may be relevant to how eosinophils adhere to and degranulate on a parasite surface or a surface coated with activated complement. Therefore the results obtained may lead to new ways of modulating the ECP release in vivo in hypereosinophilic states characterized by tissue damage which may be mediated by ECP.

Adherence of eosinophils to an antibody coated surface (antialbumin-albumin-Sepharose) was investigated. Some Fc receptor dependent binding was demonstrated. However, also Fc-receptor negative eosinophils adhered efficiently. Therefore it is possible that Fc receptor dependency is not the most important factor for binding of eosinophils to an antibody coated surface.

In idiopathic hypereosinophilia a population of eosinophils of abnormally low density was detected. Because these eosinophils had a low ECP content, expressed increased surface receptors and increased oxygen consumption it is likely that they were activated and degranulated in the circulation. Therefore it is suggested that ECP released from such eosinophils may be responsible for the tissue damage to the heart and other organs in this disease.

Purified ECP revealed a considerable microheterogeneity; several peptides with almost identical amino acid composition were obtained by high resolution chromatography. The amino terminal sequence was established for one peptide. No significant homology was found with polypeptides of known amino acid sequence indicating that ECP is a protein unique for the eosinophil. Further information on structural modifications of ECP was obtained by studies of ECP biosynthesis using marrow cells from patients with eosinophilia. Biosynthetic labeling demonstrated biosynthesis of ECP with a molecular weight of 22,000, which was slowly processed to lower molecular weight ECP corresponding to granule-bound ECP. Future determination of the nucleotide sequence for ECP may be of importance to define the functions of ECP and the role of eosinophils.

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Variations of Cationic Proteins from Eosinophil Leukocytes in Food Intolerance and Allergic Rhinitis

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Challenge tests were performed in patients with food intolerance and allergic rhinitis to evaluate the usefulness of measurement of the eosinophil cationic protein (ECP) of serum to distinguish different allergic reactions. In one group of patients with food intolerance symptom-induced challenge resulted in a marked decrease of serum-ECP. The number of blood eosinophils decreased simultaneously in some but not all of these patients. In another group of patients with food intolerance serum-ECP displayed peak-like increases followed by a decrease. The decrease in serum-ECP may reflect that consumption of ECP is a result of idiosyncrasy in the target organ. In allergic rhinitis some patients showed an initial peak-like increase of serum-ECP, which was abolished by pretreatment with disodium-cromoglycate but not by pretreatment with antihistamine. Similar results have previously been demonstrated for allergic asthma. The difference obtained in serum-ECP upon challenge in typical reagin-mediated allergy and food intolerance may indicate that the latter is not reagin-mediated. However, the interpretation of data is difficult because of lack of knowledge regarding the turnover in the circulation of ECP.

Key words: allergic rhinitis; eosinophils; eosinophil cationic protein; food intolerance.

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CLINICAL ASPECTS

Hypersensitivity reaction to food is well recognized but the diagnosis of food allergy or food intolerance is often difficult to establish. The challenge test with simultaneous measurement of ECP seems a valuable method, and the test results also may give information regarding the type of reaction involved. The test also makes it possible to evaluate the protective effect of different drugs.

The test may also be used for diagnosing and evaluating the effect of treatment in other kinds of hypersensitivity reactions like allergic rhinitis.

According to one prevailing concept the eosinophil leukocyte is regarded as a regulatory cell directed to limitation of reactions elaborated by mast cell-derived mediators of the immediate hypersensitivity reaction (4). This theory is based on the association between eosinophilia and allergy and the finding of inactivation of mast cell products by eosinophil agents (4). One agent of human eosinophils is the eosinophil cationic protein (ECP) (5), which can be detected in serum by a radioimmunoassay (6). Healthy individuals show a correlation between serum-ECP and eosinophil counts but, for in-

stance in bronchial asthma, a lower than expected level of serum-ECP is found most likely to indicate consumption of ECP (7). Treatment with beta-2-adrenergic drugs may, however, result in low levels of ECP (2). The present work was done to evaluate changes of serum-ECP during challenge test in patients with food intolerance and allergic rhinitis. Such studies were thought to give information on the possible release and consumption *in vivo* of ECP and, subsequently, the involvement of eosinophils in allergic reactions.

MATERIALS

Eighteen patients (four males and 16 females) with a history indicating intolerance against cow's milk were studied. The age range was 24–55 years. Patients were selected with regard to a history indicating intolerance to cow's milk. They had a normal lactose tolerance test but described discomfort when drinking cow's milk. Gastrointestinal symptoms – diarrhea, abdominal pain, distention – dominated, but some also described extraintestinal symptoms such as headache, arthralgia, eczema, etc. Regarding serum-IgE-concentration and RAST against cow's milk – see Results.

The controls consisted of two males and one female without any history indicating food intolerance.

Five patients with seasonal allergic rhinitis due to grass pollen were also studied. They all had positive skin and ophthalmic tests for the allergen.

METHODS

The patients with a history indicating food intolerance and the controls were free of milk for 6 days. A challenge test was then performed with 150 ml cow's milk. Blood was taken via a venous catheter before challenge and 15, 30, 60 min, 2, 4, 8 h after the challenge. The number of blood eosinophils were measured in a Fuchs-Rosenthal chamber using the method of Forsham et al. (3). Serum was collected within 2 h and stored at -17°C until assayed for ECP.

The patients with allergic rhinitis were challenged by application of a pollen extract in the conjunctival sac. In order to reduce symptoms some were challenged after oral pretreatment with antihistamine. Dexchlorpheniraminum (Polaramin) was given – 2 mg 12 h and 2 mg 2 h before the challenge. One patient was challenged after oral pretreatment with disodium cromoglycate (Lomudal®) 200 mg $\times$ 4 for 2 days. Cromoglycate was not applied locally in the conjunctival sac. Blood was taken via a venous catheter before and every 15 min after the challenge.

S-ECP was measured by a radioimmunoassay as previously described (6).

RESULTS

Seven of the 18 patients with a history of intolerance to cow's milk showed a decrease in serum-ECP and to some extent also in eosinophil counts upon challenge with milk (Fig. 1). The lowest levels for serum-ECP (approx. 30% of initial value) were seen after 2 h. All these patients showed gastrointestinal symptoms within 2–3 h of the challenge.

In five of the 18 patients with milk intolerance the challenge produced a decrease in the eosinophil count, but serum-ECP displayed peak-like increases within 2 h followed by a decrease (Fig. 2). These patients showed late symptoms (after 4 h or later) often from extraintestinal organs, e.g. arthralgia.

Only one of these 12 patients had an elevated serum-IgE-concentration. Eight of the patients were tested for IgE antibodies against cow's milk (RAST) and were negative.

Six of the 18 patients with a history of intolerance to milk, as well as three healthy individuals, showed no significant changes of serum-ECP or eosinophil counts (data not shown), nor any symptoms upon challenge.

Four of five patients with allergic rhinitis showed a more or less profound peak-like increase of serum-ECP approximately 1 h after challenge with grass pollen (Fig. 3). Data for eosinophil counts showed no consistent variations. Pretreatment with disodium cromoglycate abolished the peak-like increase of serum-ECP upon challenge (Fig. 4). Pretreatment with antihistamine did not abolish the increase of serum-ECP upon challenge, but it eventually seemed to be delayed (Fig. 4).

DISCUSSION

Recent work has produced some new information on the properties of eosinophil leukocytes with regard to granule content, cell surface receptors and molecular interactions *in vitro* (4). There is a great need for methods to study eosinophil behaviour *in vivo*. One possibility

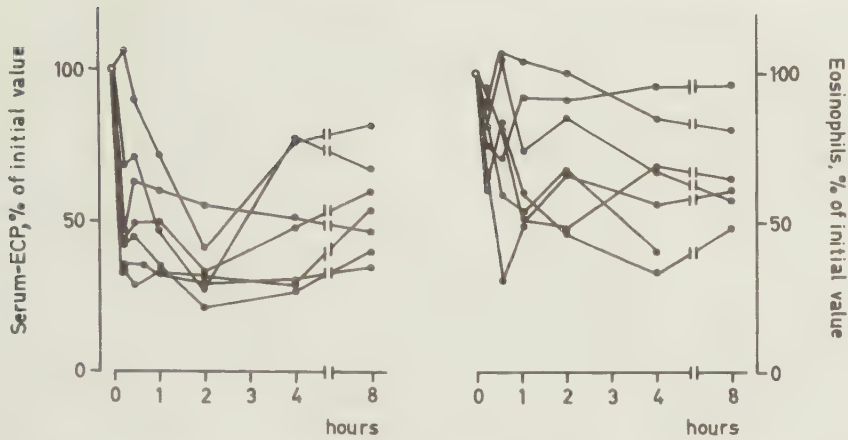


Fig. 1. Serum-ECP and eosinophil counts during challenge tests with milk in seven patients who showed a decrease of serum-ECP upon challenge. Initial levels of serum ECP varied between 8–59 $\mu\text{g/l}$. Initial eosinophil counts varied between 62–424 cells/ μl blood.

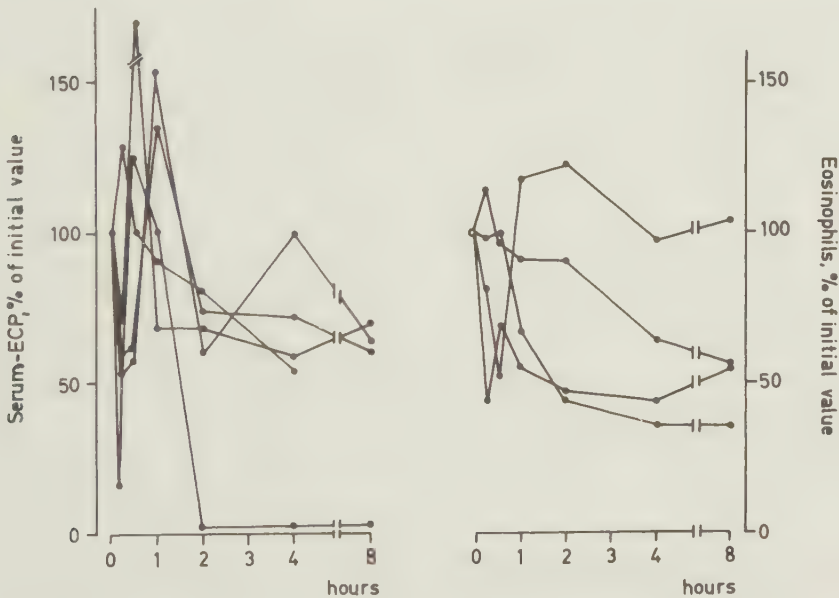


Fig. 2. Serum-ECP and eosinophil counts during challenge tests with milk in five patients who showed an initial increase of serum-ECP followed by a decrease upon challenge. Initial levels of serum-ECP varied between 4–12.5 $\mu\text{g/l}$. Initial eosinophil counts varied between 53–184 cells/ μl blood.

available is to monitor variations of the serum content of the specific eosinophil product, ECP. This study has shown that changes occur in serum-ECP and eosinophil counts during symptom-inducing challenge in food allergy. One consistent pattern was a marked decrease of serum-ECP which did not correlate directly

with the number of blood eosinophils. In addition, some patients demonstrated an early peak-like rise of serum-ECP similar to our findings in challenge of patients with allergic rhinitis.

The interpretation of data on serum-ECP is complicated because of the lack of knowledge

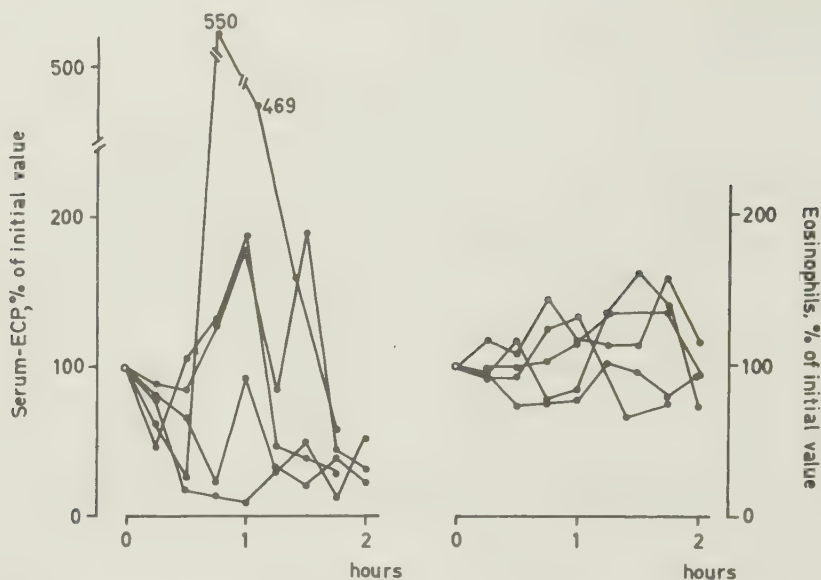


Fig. 3. Serum-ECP and eosinophil counts during challenge with local administration of grass pollen in five patients with allergic rhinitis. Initial levels of serum-ECP varied between 11–70 $\mu\text{g/l}$. Initial eosinophil counts varied between 69–1020 cells/ μl blood.

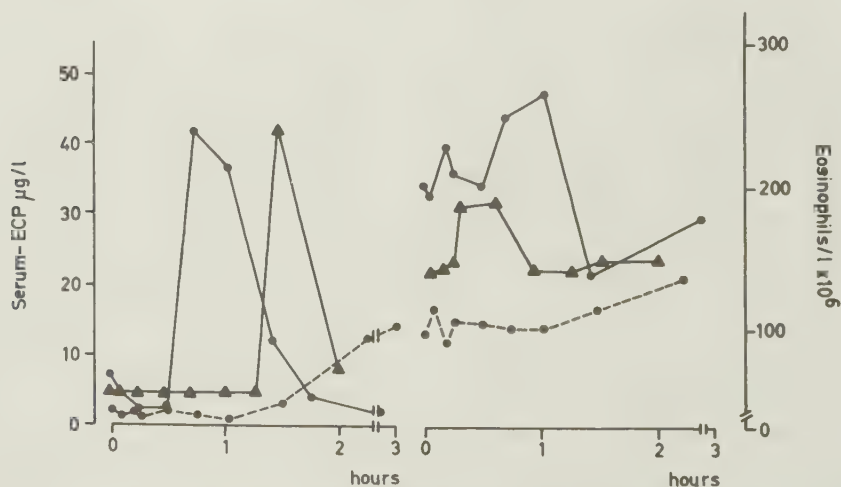


Fig. 4. Serum-ECP and eosinophil counts during challenge with grass pollen (●—●) in a patient with allergic rhinitis who had been pretreated with disodium cromoglycate (●---●) or antihistamine (▲—▲).

about the normal rate of turnover in the circulation of ECP. It is not known if all serum-ECP derives from circulating eosinophils as these represent only a minor part of the total eosinophil mass. Changes in binding proteins with consequences for the elimination could occur during pathological conditions. Nevertheless,

the decrease of serum-ECP found during challenge in food allergy most likely depends on an increased rate of elimination and perhaps consumption in the target organ. The decrease in blood eosinophils occurring simultaneously may also be partly responsible for a decreased serum-ECP. Challenge in asthma patients by

inhalation of specific allergen often resulted in an early peak-like increase of serum-ECP (1). Such a rapid change of serum-ECP must result from release from circulating eosinophils or eosinophils activated in the lung; it occurred in spite of a simultaneous decrease of blood eosinophil counts. Initial peak-like increases of serum-ECP were seen only in a few patients with food intolerance and these usually had no immediate symptoms upon challenge but late symptoms instead. Food intolerance with early symptoms was seen only in patients with a decrease in serum-ECP. It is possible that these early symptoms do not depend on reagin immediate type hypersensitivity but on some type of reaction of idiosyncrasy. It should be mentioned that patients with symptoms from intrinsic asthma (aspirin idiosyncrasy) have very low levels of serum-ECP (7). It is possible that there is no early release of ECP from eosinophils in idiosyncrasy but only consumption of ECP. Peak-like increases of ECP in reagin-mediated allergic reactions may depend on the formation of immune complexes which either locally or in the circulation induce exocytosis of ECP.

Allergic rhinitis is a reagin-mediated immediate type hypersensitivity reaction. In spite of the small size of the target organ some patients showed a clear peak-like increase of serum-ECP upon challenge. Both the symptoms and the peak-like increase of serum-ECP were abolished by pretreatment with disodium cromoglycate, indicating that changes of serum-ECP are somehow dependent on mast cell degranulation. On the other hand, pretreatment with antihistamine only abolished the symptoms and not the peak-like increase of serum-ECP, even if it was perhaps delayed (because of the absence of a histamine-induced increase of permeability?).

These studies have suggested that serum-ECP may be useful for distinguishing certain types of allergic reactions, but interpretation of the data is complicated due to the lack of knowledge regarding the turnover of serum-ECP.

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Mechanisms for eosinophil degranulation; release of the eosinophil cationic protein

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Summary. Mechanisms for degranulation in human eosinophils were evaluated. Release of eosinophil cationic protein (ECP), a unique eosinophil granule constituent, was measured upon exposure of purified eosinophils to a large surface consisting of Sephadex beads coated with serum, which leads to complement activation. Extracellular release of approximately 15% of the cellular ECP occurred both with eosinophils from patients with eosinophilia and normal people. Almost all eosinophils isolated from patients with eosinophilia and normal people adhered to serum-treated Sephadex. The data suggest that interaction through C3 receptors is a prerequisite for ECP release from eosinophils when exposed to serum-treated Sephadex. Both cytochalasin B, cytochalasin D and hydrocortisone reduced the release of ECP. Neither the cytochalasins nor hydrocortisone inhibited the adherence of eosinophils to the Sephadex beads. Thus the inhibitory effect of these agents on ECP release is a direct effect on the degranulation process. ECF-A, histamine and colchicine did not affect the release mechanism. No direct relationship was found between degranulation and oxidative burst inasmuch as some soluble mediators induced a high respiratory burst without a concomitant ECP release. Our data suggest that mechanisms for degranulation are not fully identical in eosinophils and neutrophils.

INTRODUCTION

The eosinophil leucocyte is identified by its crystalloid-containing cytoplasmic granules. Unique components of the granules are proteins such as the major basic protein (MBP; Gleich, Loegering & Maldonado, 1973; Gleich *et al.*, 1974, 1976) and the eosinophil cationic protein (ECP; Olsson *et al.*, 1977), which both show toxicity for schistosomules of *Schistosoma mansoni* (Butterworth *et al.*, 1979; McLaren *et al.*, 1981) and for a variety of mammalian cells (Frigas, Loegering & Gleich, 1980; Fredens, Dahl & Venge, 1982). The reasons for the occurrence of eosinophilia during anaphylactic reactions (Beeson & Bass, 1977) and parasite diseases (Kay, 1979) are not clear. It is possible that studies of unique granule constituents such as MBP and ECP and studies of mechanisms for degranulation may lead to an understanding of the biological function of the eosinophil. We already know that ECP is actually released *in vivo* as judged by studies of serum-ECP in some allergic diseases (Dahl, Venge & Olsson, 1978; Winqvist *et al.*, 1981) and hypereosinophilic syndromes (Spry, 1980).

The present work was done to study mechanisms for degranulation in eosinophils using the release of ECP as a specific marker for that process. Release was induced by exposure of purified eosinophils to a large surface consisting of Sephadex beads coated with serum. Eosinophils were isolated from both healthy individuals and patients with eosinophilia. The results show that the mechanism for release depends on

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interaction of the eosinophil with a large surface coated with activated complement. A respiratory burst elicited with certain soluble factors occurred without concomitant degranulation. Pharmacologic modulation indicated somewhat different mechanisms for degranulation in eosinophils and neutrophils.

MATERIALS AND METHODS

Chemicals

Hanks's balanced salt solution (HBSS) was from Flow Laboratories, England. Cytochalasin B and cytochalasin D from Calbiochem-Behring Corp., La Jolla, CA, were dissolved in dimethyl sulphoxide (DMSO) and diluted for use in HBSS to a final maximal DMSO concentration of 0.5%. Calcium ionophore A23187 was from Calbiochem-Behring Corp., La Jolla CA, issued by Eli Lilly Corp. The following chemicals were obtained from Sigma Chemical Corp., St Louis, MO: hydrocortisone-21-sodium succinate, colchicine, histamine, phorbol-12-myristate-13-acetate (PMA), zymosan A, concanavalin A. The synthetic eosinophil chemotactic factor of anaphylaxis (ECF-A, L-Val-Gly-Ser-Glu) was a gift from Dr Edward Goetzl, Robert B. Brigham Hospital, Boston, MA. Sephadex G-15, Dextran 250, Percoll (polyvinylpyrrolidone-coated silica gel) and concanavalin A Sepharose B (Con A Sepharose) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Trypsin was from Kabi, Stockholm, Sweden. Cetyltrimethyl ammoniumbromide (CTAB) was from BDH, Poole, U.K. Polypropylene tubes were from Falcon Plastics, Los Angeles, CA. Human serum was stored at -80° until used unless otherwise stated.

Isolation of eosinophils by density gradient centrifugation

Heparinized blood was collected from healthy volunteers with <300 eosinophils per μ l blood and from volunteers with eosinophilia (filariasis, ancylostomiasis, primary hepatocellular carcinoma, eosinophil gastroenteritis, lymphoma).

Five parts of blood were mixed with one part of 6% Dextran 250 in 0.15 M NaCl and left at room temperature 45 min for red cell sedimentation. The dextran-plasma was collected and centrifuged at 450 g for 5 min and the cells obtained were washed twice in HBSS.

A density gradient was made using a modification of Gärtner (Gärtner, 1980; Winqvist *et al.*, 1982). Percoll

and HBSS were mixed to obtain solutions with different densities, pH 7.4. It was important to adjust the osmolarity carefully to 275 mosm/l, otherwise no separation was achieved. Gradients were formed using a peristaltic pump (Minipuls II, Gilson, France) and consisted of Percoll solutions with the following densities: 1.110 (1 ml), 1.095 (3 ml), 1.090 (3 ml), and 1.085 (3 ml), layered on top of each other in 17×100 mm polypropylene tubes. Two millilitres of cells (25×10^6 /ml) suspended in Percoll, 1.075 g/ml, were layered on each gradient. The tubes were centrifuged in an angle rotor (angle 45°) at 1600 g for 20 min at room temperature. One-millilitre fractions were collected from the bottom of the tubes using the peristaltic pump. The density of each fraction was measured, the cells were washed in HBSS and counted. Cyto-centrifuge smears were prepared for differential counts. The cell suspensions obtained contained 64–98% eosinophils.

Induction of eosinophil degranulation

Serum-treated Sephadex beads. One-tenth of a millilitre of Sephadex beads (0.3×10^6 /ml) suspended in HBSS was preincubated at 37° for 10 min with 0.1 ml human serum and an aliquot of 0.1 ml eosinophils (0.3×10^6) was added. After incubation at 37° the reaction was terminated by centrifugation for 1.5 min in a Beckman Microphuge (Beckman Instruments, Palo Alto, CA) at 11,600 r.p.m. (8370 g) at room temperature. The supernatant was removed and mixed with CTAB to a final concentration of 0.3%. The cell pellet was extracted in 0.3% CTAB, centrifuged at 8370 g and the resulting supernatant was used for ECP assay. Samples were stored frozen until assayed. Also for experiments described below the reaction was terminated in this way.

In some experiments the eosinophils were incubated at 37° with cytochalasin B or D (10μ g/ml) for 15 min, with hydrocortisone (10^{-5} M) for 30 min, with colchicine (10^{-5} M) for 30 min, with histamine (10^{-5} M) for 30 min or ECF-A (10μ g/ml) for 40 min before addition of Sephadex beads.

Zymosan-treated serum (ZTS) was prepared by suspending zymosan (5 mg/ml) in 50% serum followed by incubation at 37° for 15 min. After centrifugation, 0.2 ml of the supernatant (ZTS) was immediately mixed with 0.1 ml eosinophil suspension (0.3×10^6) at 37° .

Concanavalin A Sepharose was washed four times and suspended in HBSS. The suspension, 0.1 ml, was mixed with 0.1 ml serum and 0.1 ml cells (0.3×10^6 eosinophils) at 37° . The final Con A Sepharose concentration was 100 $\mu\text{g/ml}$ Con A. In some experiments, soluble Con A diluted in HBSS was used.

Immune complexes. Antibodies against human serum albumin (HSA; Kabi, Stockholm, Sweden) were raised in rabbits. Unfractionated antiserum was mixed with HSA (1 mg/ml) in ratios tested to result in optimal immune complex formation and left at $+4^\circ$ overnight. The precipitate was washed three times in saline and the protein concentration was determined according to Lowry *et al.* (1959). The immune complexes were resuspended to a concentration of approximately 3 mg/ml protein. One-tenth of a millilitre of this suspension, diluted to 0.75 mg/ml, and 0.1 ml serum or 0.1 ml HBSS was after pre-incubation at 37° for 10 min mixed with the cells.

Induction of neutrophil degranulation

Neutrophils (purity $>90\%$) were obtained from the same density gradient as the eosinophils. They were suspended in HBSS, $3 \times 10^6/\text{ml}$, and 0.1 ml of the suspension was mixed with serum-treated Sephadex as described above for eosinophil degranulation.

Assay for eosinophil cationic protein (ECP)

ECP was measured by a radioimmunosorbent assay (Venge, Roxin & Olsson, 1977). The range of the standard curve was 1.95–500 ng/ml. The supernates were diluted 10–25 times.

Assay for myeloperoxidase (MPO) and lactoferrin

MPO and lactoferrin were determined with immunoradiometric assay (Olsson *et al.*, 1979). The range of the standard curves was 5–60 ng/ml. The supernates were diluted 15 times.

Oxygen consumption

Sephadex beads (0.8 ml; $0.3 \times 10^6/\text{ml}$ HBSS) were incubated with 0.7 ml serum at 37° for 10 min and mixed with 1 ml of a cell suspension to a final concentration of 4×10^6 eosinophils/ml. Thereafter oxygen consumption was measured with a Clark electrode mounted in a 2.24-ml glass-stoppered incubation chamber equipped with magnetic stirring and heating to 37° (Eschweiler & Co, Kiel, Germany; Winqvist *et al.*, 1982).

In some experiments eosinophils were incubated at

37° with cytochalasin B (10 $\mu\text{g/ml}$) for 15 min or ECF-A (10 $\mu\text{g/ml}$) for 40 min before addition of serum treated Sephadex.

Oxygen consumption of eosinophils was measured also after addition of ZTS or PMA (100 ng/ml).

Adherence assay

One millilitre of Sephadex G-15 was incubated with normal serum for 10 min at 37° and packed in a 2-ml syringe. Aliquots of 0.4 ml eosinophils were added to the columns, which were incubated for 15 min at 37° . The effluents were then collected and eosinophils counted. To study the effect on adherence eosinophils were preincubated with hydrocortisone (10^{-5} M for 30 min) and cytochalasin B (10 $\mu\text{g/ml}$ for 15 min).

Trypsinization of cells

The eosinophils, $3 \times 10^6/\text{ml}$, were incubated with 0.1% trypsin for 30 min at 37° and washed with HBSS.

RESULTS

Exposure of eosinophils to Sephadex beads coated with serum resulted in extracellular release of approximately 15% of the cellular content of ECP (Fig. 1). No difference was found in degranulation between eosinophils isolated from patients with eosinophilia as

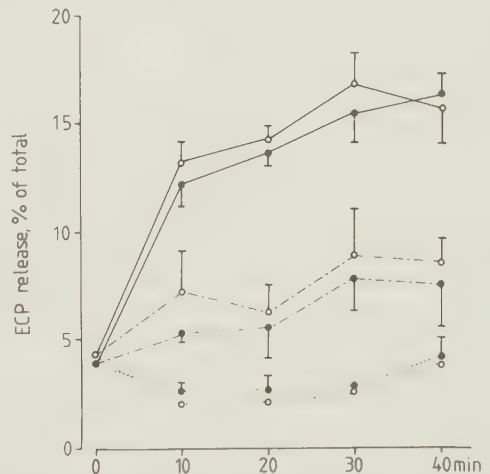


Figure 1. Release of ECP % ($\pm$ SEM) of total ECP content, with serum-treated Sephadex from eosinophils of healthy individuals (●—●) and eosinophils of patients with eosinophilia (○—○). ECP release is also shown after pretreatment of the cells with cytochalasin B, 10 $\mu\text{g/ml}$ (— — —), or cytochalasin D, 10 $\mu\text{g/ml}$ (— · — · —)

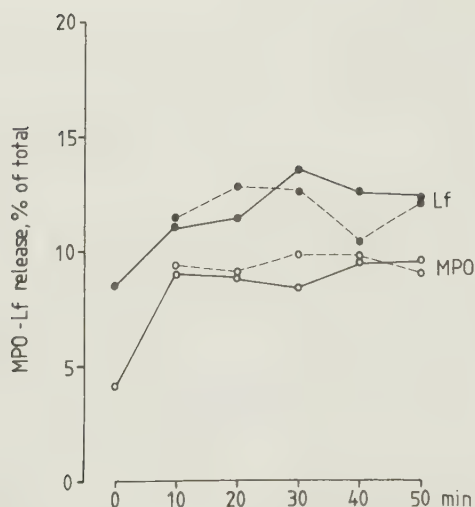


Figure 2. Release (% of total content) of myeloperoxidase (MPO) and lactoferrin (Lf) from neutrophils with serum-treated Sephadex (—●—). Release from cells preincubated with cytochalasin B, 10 μ g/ml, is also shown (---○---).

compared to eosinophils from normal individuals. Cytochalasin B (10 μ g/ml), and cytochalasin D (10 μ g/ml) both inhibited the ECP-release, cytochalasin D being the most potent inhibitor.

For comparison, neutrophil release of a primary granule constituent (myeloperoxidase) and a secondary granule constituent (lactoferrin) were measured upon exposure of neutrophils to serum-coated Sephadex beads (Fig. 2). The relative release of myeloperoxidase and lactoferrin was lower than the relative ECP release from eosinophils and, contrary to ECP release, it was not inhibited by cytochalasin B.

ECP release was reduced when eosinophils were exposed to Sephadex beads pretreated with heat-inactivated serum instead of fresh serum (Fig. 3). Untreated Sephadex did not induce degranulation at all. Furthermore, trypsinized eosinophils showed a decrease in ECP-release during incubation with serum treated Sephadex. These results suggest that interaction of eosinophils with serum-treated Sephadex through C3-receptors is a prerequisite for degranulation since C3-receptors on the cell surface are destroyed by trypsin (Tai & Spry, 1980).

Hydrocortisone reduced the Sephadex-stimulated ECP release (Fig. 4). Other pharmacological agents tested, such as colchicine, ECF-A and histamine, did not seem to affect the ECP release (data not shown). Immune complexes consisting of albumin-anti-albu-

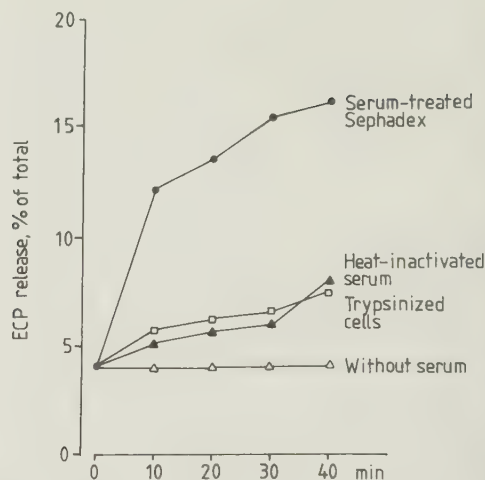


Figure 3. Release of ECP, % of total, with serum-treated Sephadex (—●—), Sephadex treated with heat-inactivated (56°) serum (—▲—), untreated Sephadex (—△—) and with serum-treated Sephadex after trypsinization (0-1%, 30 min) of the cells (—□—).

min also induced release of ECP from eosinophils, provided that fresh serum was present (Fig. 5). Soluble factors such as zymosan-treated serum (ZTS), Con A and PMA did not by themselves induce ECP release

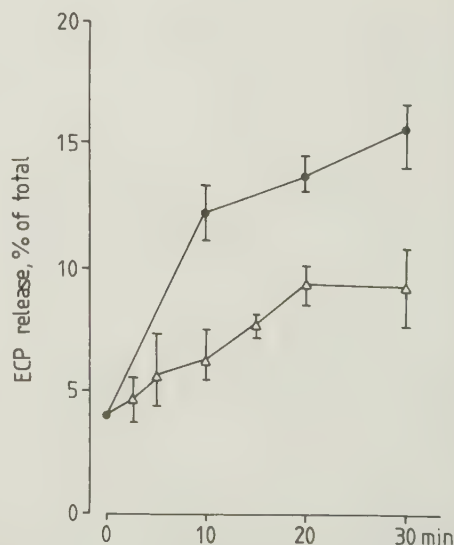


Figure 4. Effect of preincubation of eosinophils with hydrocortisone (10^{-5} M, 30 min) on release of ECP, % ($\pm$ SEM) of total, with serum-treated Sephadex (—△—). Untreated cells (—●—).

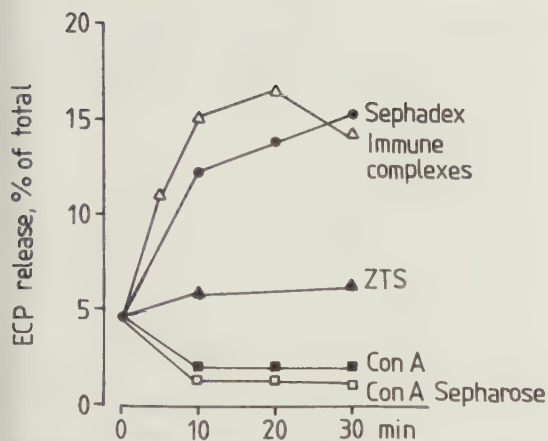


Figure 5. Release of ECP, % of total from eosinophils incubated with: (●—●) serum-treated Sephadex; (△—△) immune complexes (albumin-anti-albumin); (▲—▲) zymosan-treated serum (ZTS); (■—■) Con A, 100 μ g/ml; (□—□) Con A Sepharose, 100 μ g/ml.

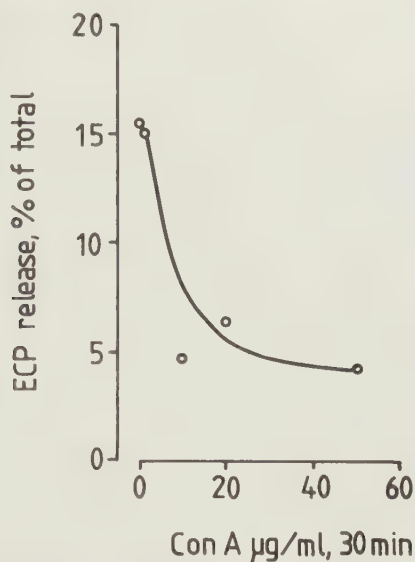


Figure 6. Effect of preincubation of eosinophils with Con A (0.1–50 μ g/ml, 30 min) on release of ECP, % of total, upon incubation with serum-treated Sephadex.

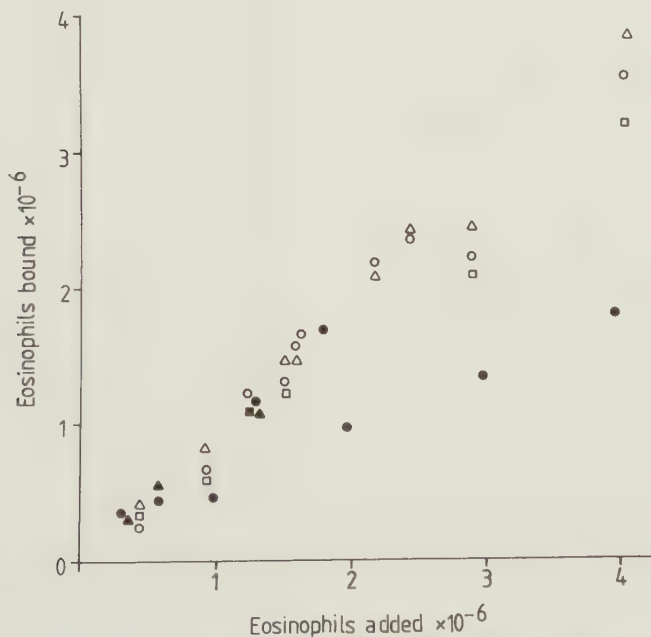


Figure 7. Adherence of eosinophils to serum-treated Sephadex. The number of eosinophils added to the Sephadex was varied and the number of eosinophils bound was calculated. Eosinophils obtained from four patients with eosinophilia (○) and three healthy individuals (●) were studied. Results are also presented for eosinophils pretreated with cytochalasin B (△,▲) and hydrocortisone (□,■); open symbols represent eosinophils from patients with eosinophilia and closed symbols represent eosinophils from normal people.

(Fig. 5); nor did Con A Sepharose. Con A, however, reduced the Sephadex-stimulated release of ECP (Fig. 6).

Almost all the eosinophils isolated from patients with eosinophilia and normal people adhered to serum-treated Sephadex (Fig. 7). Neither cytochalasin B nor hydrocortisone inhibited the adherence of the cells.

No correlation was found between the respiratory burst measured as oxygen consumption and the ECP release. In particular ZTS and PMA, which both elicited a marked respiratory burst (Fig. 8), did not induce degranulation. Actually, incubation of eosinophils with opsonized Sephadex produced only a slight increase in oxygen consumption. Pretreatment of the cells with ECF-A increased and cytochalasin B decreased the Sephadex stimulated respiratory burst.

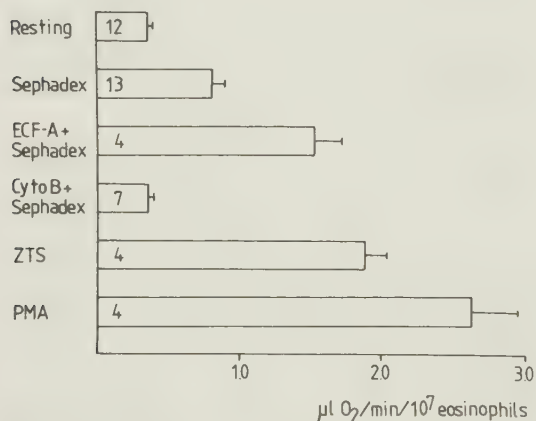


Figure 8. Oxygen consumption, $\mu\text{l O}_2/\text{min}/10^7$ eosinophils, in resting eosinophils as well as after incubation with serum-treated Sephadex, ECF-A plus serum-treated Sephadex, cytochalasin B plus serum-treated Sephadex, zymosan-treated serum (ZTS) or PMA (100 ng/ml). The number of experiments is indicated in the figure. Bars indicate SEM.

DISCUSSION

The discovery of ECP (Olsson *et al.*, 1977) has provided a tool for studies of release of a specific eosinophil constituent as a result of degranulation. Furthermore, development of methods for purification of eosinophils has made it possible to study eosinophils from healthy individuals. The present study showed that eosinophils release ECP when exposed to a large surface (Sephadex) coated with activated complement (Metcalfe *et al.*, 1977). Adher-

ence of eosinophils to serum-treated Sepharose also results in release of arylsulphatase (Metcalfe *et al.*, 1977). Phagocytosis of opsonized zymosan by eosinophils results in release of histaminase, arylsulphatase and β -glucuronidase (Zeiger & Colten, 1977). Our results suggest some differences in mechanisms for induction of degranulation in eosinophils and neutrophils. Despite the fact that eosinophils exhibit fewer receptors for C3b than neutrophils (Anwar & Kay, 1978), the former cell shows higher granule protein release on opsonized Sephadex than the latter.

Degranulation of neutrophils can be induced by a number of mechanisms such as lysis, regurgitation during phagocytosis, reversed endocytosis and soluble factor-induced secretion (Goldstein & Weissman, 1979). Of these mechanisms, reversed endocytosis may be the most important for eosinophils; serum-coated Sephadex used in the present study provides an opsonized surface too large for ingestion and induces extracellular release of granule constituents. An identical mechanism may operate on the surface of opsonized parasites leading to eosinophil degranulation and parasite killing.

Neutrophils are known to respond to soluble C5a with extracellular secretion from azurophil and specific granules provided that the cell has been treated with cytochalasin B (Goldstein & Weissman, 1979). Contrary to this, eosinophil degranulation on exposure to serum-treated Sephadex was reduced both by cytochalasin B and cytochalasin D. Zymosan-induced histaminase release from eosinophils, but not from neutrophils, was also reduced in the presence of cytochalasin B (Zeiger & Colten, 1977). Cytochalasin B interferes with the function of cytoplasmic microfilaments and also inhibits hexose transport (Zigmond & Hirsch, 1973) but cytochalasin D only affects microfilaments (Miranda *et al.*, 1974a, b).

Cytochalasin B reversibly inhibited binding of eosinophils to IgG antibody-coated red cells but not to red cells coated with C3b (Tai & Spry, 1980). Therefore treatment of eosinophils with cytochalasins was not expected to interfere with binding to serum-treated Sephadex. Actually, we found no inhibition of eosinophil adherence upon treatment with cytochalasins. These agents seem to inhibit directly the degranulation process in eosinophils. Also hydrocortisone reduced the ECP release without interfering with adherence of the eosinophils. Oral prednisone administration has been shown to reduce whole blood eosinophil adherence but isolated eosinophils had normal adherence both after oral prednisone administration and after

addition of hydrocortisone *in vivo* (Altman *et al.*, 1981).

Con A and PMA provoke secretion from neutrophil-specific granules (Goldstein & Weissman, 1979) but these agents did not provoke ECP-release from eosinophils. It was, however, reported that Con A gives rise to perinuclear vacuoles containing granule contents in eosinophils but without release to the exterior (Tai & Spry, 1981). Ultrastructural changes were most marked in granule crystalloid material. In the light of our own results on inhibition of ECP release with cytochalasins, it is of interest that cytochalasin B was found to inhibit the effects of Con A-induced vacuolization (Tai & Spry, 1981). On the other hand, we found that pretreatment of the cells with Con A reduce the Sephadex-stimulated ECP release. This effect may depend on either inhibition of adherence or degranulation.

Eosinophil chemotactic factor of anaphylaxis (ECF-A) has been reported to enhance the expression of human eosinophil receptors for C3b (Anwar & Kay, 1978) and to initiate release of arylsulphatase (Wasserman, Goetzel & Austen, 1975). We did not find a similar effect for release of ECP, which may depend on different compartmentalization of arylsulphatase and ECP. We, however, used an ECF-A tetrapeptide and Wasserman utilized a crude Sephadex G-25 preparation.

We have clear evidence that ECP can be released *in vivo* detected as an increased level of serum-ECP. This was found for example during antigenic challenge in certain allergic diseases (Dahl *et al.*, 1978; Winqvist *et al.*, 1981). It is not clear if increased serum-ECP is the result of release from circulating eosinophils or eosinophils activated in the tissues. Circulating immune complexes could induce some degranulation which is supported by the present finding of ECP release upon interaction between eosinophils and immune complexes. It is, however, more likely that ECP is released in the tissues upon exposure to surfaces coated with immunoglobulin and complement with the escape of some ECP to the circulation.

Eosinophils from patients with eosinophilia have been reported to have a decrease of cell surface charge, activation of acid phosphatase and an increase in membrane hexose transport when compared with normal eosinophils (Bass *et al.*, 1980). We found, however, no difference in ECP release between such eosinophils and eosinophils from healthy individuals.

The finding of release of only 15% of total ECP content was not a result of adherence and degranula-

tion of only a fraction of eosinophils since almost all eosinophils added adhered to serum-treated Sephadex.

No direct relationship was found between degranulation and oxidative burst inasmuch as some soluble mediators induced a high respiratory burst in eosinophils without a concomitant ECP release. Studies with non-phagocytic stimuli indicate that these two phenomena can occur independently in neutrophils too (Goldstein & Weissman, 1979). It is most evident with C5a, which in the absence of cytochalasin B stimulates neutrophils to generate superoxide without provoking enzyme release (Goldstein & Weissman, 1979). Contrary to findings with neutrophils (Goldstein & Weissman, 1979), pretreatment of eosinophils with cytochalasin B decreased Sephadex-stimulated enhancement of the oxidative metabolism. It is emphasized that this effect is not caused by inhibition of eosinophil adherence since that process was unaffected by cytochalasin.

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EFFECT OF ZINC AND OTHER CATIONS ON THE RELEASE OF THE EOSINOPHIL CATIONIC
PROTEIN

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Running head: effect of zinc on eosinophils.

SUMMARY

The eosinophil cationic protein, ECP, is a unique eosinophil granule constituent, which is released extracellularly after exposure of the eosinophils to a non-phagocytosable surface such as complement-coated Sephadex beads. ECP is released to some extent even in the absence of Ca^{2+} and Mg^{2+} , but both these cations augment the release reaction tested alone and an optimal release is observed only in the presence of 2 mM Ca^{2+} and 2 mM Mg^{2+} in the medium. Zn^{2+} at concentrations from 0.25 - 4.0 mM inhibited the release of ECP in a dose-dependent fashion, with or without Ca^{2+} and Mg^{2+} in the medium. Mn^{2+} had dual effects, stimulating the ECP-release in the absence of Mg^{2+} and Ca^{2+} , and inhibiting the release in the presence of these cations. Li^{1+} caused minor inhibition of ECP-release but only in the absence of Ca^{2+} and Mg^{2+} . The inhibitory effect of Zn^{2+} was immediate and reversible after washing of the cells suggesting that the inhibition is due to interaction with the plasma membrane functions.

Key words: eosinophil degranulation - effect of zinc, calcium and magnesium.

INTRODUCTION

The eosinophil possesses two distinct types of granules. The larger granules have an electron-dense crystalloid matrix and contain eosinophil peroxidase, β -glucuronidase, histaminase, cationic proteins and eosinophil-derived neurotoxin (1). These granules are responsible for the peculiar affinity for acid dyes such as eosin. The smaller granules are of an amorphous nature and are rich in arylsulfatase and acid phosphatase (2). The cell membrane of the eosinophil contains lysophospholipase, which spontaneously form structures termed Charcot-Leyden crystals (3, 4).

Zinc has been localized within the matrix of the crystalloid-containing granules of rat and cattle eosinophils by an electron-microscope histochemical technique (5). Granules isolated from cattle eosinophils contained 3×10^{-16} gm of zinc per granule. The Charcot-Leyden crystals contain an appreciable quantity of zinc (188 parts per million) (6, 7). One of the cationic proteins (eosinophil cationic protein, ECP) contains approximately 2.5 moles of Zn per mole protein (8, 9). The eosinophil and neutrophil leukocytes are supposed to have the same total zinc content (10).

Zn^{2+} selectively inhibits the release of collagenase from the neutrophil primary granules (11). Another cation, Mn^{2+} , also reduces the release of collagenase and in addition other primary granule constituents such as myeloperoxidase, chymotrypsin-like cationic protein and lysozyme (11).

It has also been reported that Zn^{2+} inhibits the release of histamine by stimulated tissue mast cells (12), granulocyte chemotaxis and phagocytosis (13) and platelet contraction, release and aggregation (13).

We have previously shown that the eosinophil cationic protein, ECP, is released from eosinophils when the cells are challenged with serum-coated Sephadex beads and we have characterized some of the requirements for the release of ECP (14). In this report we describe the dependency on Ca^{2+} and Mg^{2+} for the release of ECP and show that Zn^{2+} inhibits and other cations modulate the release of ECP.

MATERIALS AND METHODS

Isolation of eosinophils

Heparinized blood was collected from healthy volunteers and mixed with 6% Dextran 250 in 0.15 M NaCl to a final concentration of 1% dextran. The red cells were let to sediment at 37°C for 45 min and the leukocyte-rich supernatant collected and centrifuged for 5 min at 450 g. The cells were washed and resuspended in Hanks' balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS) and varidase (125 IE/ml). Eosinophils were isolated from a discontinuous metrizamide gradient as described by Vadas et al. (15); each gradient consisted of 1.8 ml aliquots of 18, 20, 22, 23, 24 and 25% metrizamide in 17x100 mm polypropylene tubes. Two ml cell suspension with $25\text{--}50 \times 10^6$ cells per ml were layered on top of each gradient, and the gradients were centrifuged at 1200 g for 45 min at room temperature. One ml fractions were collected from the bottom of the tubes using a peristaltic pump. The eosinophil enriched fractions contained more than 80% eosinophils. The cells were washed in phosphate buffered saline without calcium or magnesium (=PBS-0; each liter containing NaCl 8.0 g, KCl 0.2 g, KH_2PO_4 0.2 g, $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ 1.44 g and glucose 1 g, pH 7.3). The cations to be tested for effects on the release of ECP were added from 40 mM stock solutions to achieve final concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mM immediately before addition of Sephadex beads (see below).

Induction of eosinophil degranulation (14)

One-tenth of a milliliter of Sephadex beads ($0.3 \times 10^6/\text{ml}$) suspended in HBSS was preincubated at 37 °C for 10 min with 0.1 ml human serum and then washed three times in PBS without Ca and Mg (PBS-0). 0.2 ml of the washed serum-treated Sephadex beads was added to an aliquot of 0.1 ml eosinophils ($0.3 \times 10^6/\text{ml}$) to which 0.25, 0.5, 1.0, 2.0 or 4.0 mM of either Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} or Li^{1+} had been added in advance. After incubation for 20 min. at 37°C the reaction was terminated by centrifugation for 1.5 min in a Beckman Microphuge (Beckman Instruments, Palo Alto, CA) at 11,600 r.p.m. (8370 g) at room temperature. The supernatant was removed and mixed with CTAB to a final concentration of 0.3 %. Samples were stored frozen until assayed for ECP.

Assay of eosinophil cationic protein (ECP)

ECP was measured by a radioimmunosorbent assay (16). The range of the standard curve was 1.95–500 ng/ml. The supernates were diluted 10–25 times. In order to exclude the possibility that Zn^{2+} interferes with the radioimmunoassay, 2 mM Zn^{2+} was added to the standard dilution series in control experiments. The presence of Zn^{2+} did not affect the radioimmunoassay.

Chemicals

Hanks' balanced salt solution (HBSS) and fetal bovine serum (FBS) were from Flow Laboratories, England. Sephadex G15 and Dextran 250 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Metrizamide was from Nyegaard & Co, Oslo, Norway. Bovine serum albumin (BSA) and trifluoperazine were from Sigma Chemical Corp., St Louis, MO. Varidase was from Lederle, Wayne, N.J. Gelatin was from Oxoid, Basingstroke, England. Cetyltrimethylammoniumbromide (CTAB) was from BDH, Poole, England.

RESULTS

Effect of Ca^{2+} and Mg^{2+} on the release of ECP

All results obtained in 2 mM Ca^{2+} and Mg^{2+} were normalized to 100% and the other results were compared with this. In PBS-0 the release of ECP was about 60% of that obtained in presence of 2 mM Ca^{2+} and Mg^{2+} . Addition of increasing concentrations of either Ca^{2+} or Mg^{2+} from 0.25 mM to PBS-0 gradually augmented the release but it was only the combination of Ca^{2+} and Mg^{2+} that resulted in optimal conditions for ECP-release (figure 1).

Effect of Zn^{2+} on the release of ECP

Figure 2 shows that the addition of Zn^{2+} from 0.25–4.0 mM significantly reduced the release of ECP. The inhibition was stronger in PBS-0 than in PBS with Ca^{2+} and Mg^{2+} , but in both cases 2.0 and 4.0 mM Zn^{2+} caused an almost total inhibition. In PBS with Ca^{2+} and Mg^{2+} 0.25 mM Zn^{2+} inhibited the release of ECP with approximately 30%. In other experiments the cells were incubated with 2.0 mM Zn^{2+} for 5, 15 and 30 min and then washed twice before addition of serum-treated Sephadex. The inhibitory effect on ECP-release was much less pronounced under these circumstances (data not shown).

Effect of Mn^{2+} and Li^{1+} on the release of ECP

Mn^{2+} or Li^{1+} could not substitute for Ca^{2+} and Mg^{2+} when added to PBS-0 in concentrations ranging from 0.25-4.0 mM (figure 3). When Mn^{2+} was added to PBS-0 the lowest concentration, 0.25 mM, resulted in a slightly stimulatory effect on ECP-release, but with increasing concentrations this stimulation vanished. Li^{1+} added to PBS-0 caused minor inhibition of ECP-release (figure 3). When Mn^{2+} and Li^{2+} was added to PBS containing 2 mM Ca^{2+} and Mg^{2+} , only Mn^{2+} caused an inhibition of the release of ECP at 0.25-0.5 mM concentrations but was without effect at the higher concentrations tested (figure 4).

DISCUSSION

In a previous study we have shown that the eosinophil cationic protein, ECP, is released by extracellular degranulation (14). The release of ECP was indispensably dependent on serum and experiments with trypsinized cells and with heat-inactivated serum suggested that the Sephadex beads must be covered with activated complement factors to be able to induce release of ECP. A comparison with the release of myeloperoxidase and lysozyme from neutrophils showed that this reaction is not inhibited by cytochalasins B or D, whereas the release of ECP from eosinophils was almost totally blocked by these agents. It has been shown by others that neutrophils release granular proteins in response to soluble factors such as zymosan-activated serum or C5a (17). In our previous work we have shown that eosinophils differ in this respect since no release of ECP was observed after incubation with zymosan-activated serum (14). It therefore seems that there are important differences between eosinophils and neutrophils with respect to their requirements for extracellular release of granular proteins, and that one important difference is the eosinophils' dependency on surface attachment for induction of ECP-release.

Neutrophils have special requirements for opsonins and divalent cations to achieve optimal phagocytosis (18, 19), and to express extracellular release of granular proteins during phagocytosis (11). Both Ca^{2+} and Mg^{2+} were necessary to achieve optimal conditions for release of primary and secondary granule proteins from neutrophils (11); tested alone Mg^{2+} was more effective than Ca^{2+} to sustain extracellular release. In this respect the eosinophils have the same requirements for Ca^{2+} and Mg^{2+} . We found in this study that increasing concentrations of either Ca^{2+} or Mg^{2+} from 0.25-4.0 mM stimula-

ted the release of ECP from eosinophils but both cations were necessary to obtain optimal conditions for ECP-release, which suggests an additive effect of Ca^{2+} and Mg^{2+} .

It has been shown that divalent cations such as Zn^{2+} and Mn^{2+} can modulate the release of granular proteins from neutrophils (11). Zn^{2+} selectively inhibited the release of collagenase, whereas Mn^{2+} in addition to inhibition of the release of collagenase also inhibited the release of myeloperoxidase, chymotrypsin-like cationic proteins and lysozyme but stimulated the release of lactoferrin. The present study has shown that Zn^{2+} also has the capacity to totally inhibit the release of ECP from eosinophils both in the presence and absence of optimal concentrations of Ca^{2+} and Mg^{2+} . Mn^{2+} however, inhibited ECP-release only in the presence of Ca^{2+} and Mg^{2+} , and only at low concentrations of Mn^{2+} . The stimulatory effect of 0.25 mM Mn^{2+} observed when added to PBS-0 suggests that Mn^{2+} interferes with more than one mechanism involved in the release of ECP, and that the effect of Mn^{2+} is dependent on whether Ca^{2+} and Mg^{2+} is present or not. In calcium-magnesium containing PBS Li^{1+} had an effect similar to that observed with Mn^{2+} showing minor inhibition at low concentration, but Li^{1+} did not stimulate the ECP-release when added to PBS-0, which suggests that the effects of Li^{1+} are mediated over other mechanisms than Mn^{2+} . The results also suggest that the mechanisms for degranulation are different in eosinophils and neutrophils.

In addition to the inhibitory effects by Zn^{2+} on the release of granular constituents from neutrophils and eosinophils, earlier studies have shown that Zn^{2+} inhibits the release of histamine from mast cells (12), and platelet aggregation and serotonin release (13) but the mechanisms by which Zn^{2+} inhibits these reactions are poorly understood. It has been suggested that Zn^{2+} may change the plasma membrane fluidity (13, 20) or inhibit the activity of plasma membrane associated enzymes such as ATPase (13, 21) and phospholipase A_2 (13, 22). There is evidence to suggest that the inhibitory effect of Zn^{2+} on the release of ECP is due to interaction with plasma membrane events, since inhibition was immediate and much less pronounced after washing of the cells even after 30 min preincubation.

Another possible site for interaction of Zn^{2+} is calmodulin, which is known from other cell systems to regulate the function of several cytoplasmic enzymes (23). Recent investigations have led to the hypothesis that Zn^{2+} - Ca^{2+} antagonism may be the result of inhibitory effects of Zn^{2+} on the function of calmodulin (24). If the inhibitory effects of Zn^{2+} that we have observed in

this work were dependent on an inhibition of calmodulin, the effects should be able to reproduce with inhibitors of calmodulin such as trifluoperazine (25). However, preliminary experiments with 10^{-4} and 10^{-5} M trifluoperazine after 10-120 min preincubation of eosinophils did not mimic the inhibitory effects of Zn^{2+} . Therefore, it is less likely that Zn^{2+} inhibits the release of ECP via interaction with calmodulin.

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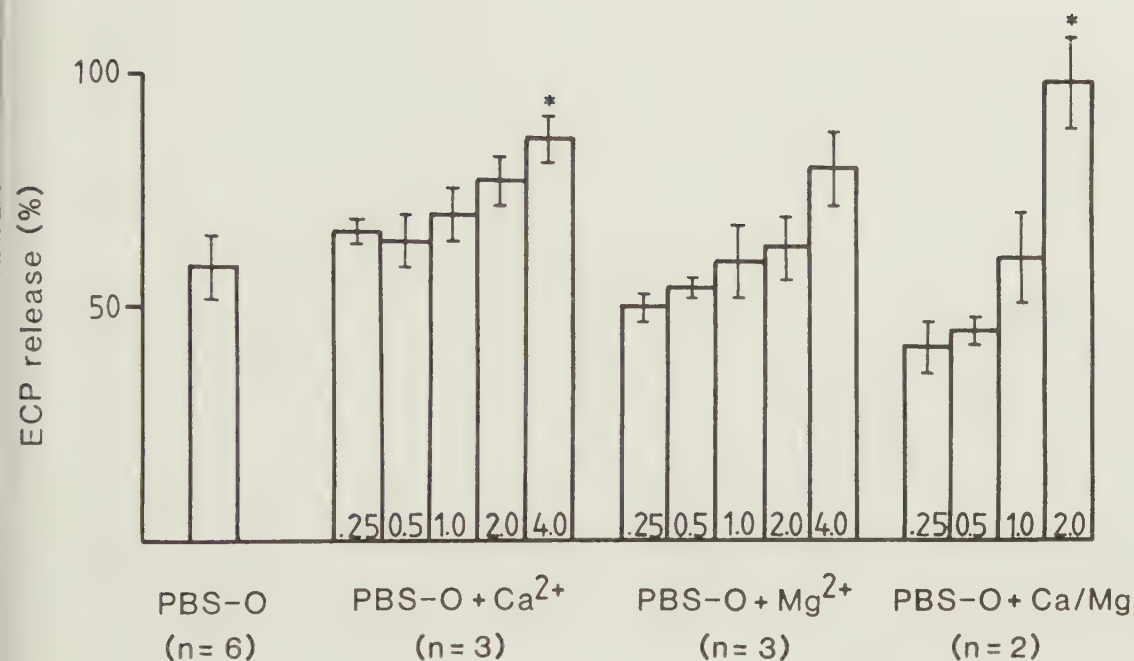


Fig. 1. Effects of Ca²⁺ and Mg²⁺ on the release of ECP from eosinophils. The results obtained with PBS containing 2 mM Ca²⁺ and 2 mM Mg²⁺ (PBS) were normalized to 100% and all other results are compared with this normalized value. PBS-O is PBS without Ca²⁺ and Mg²⁺. Increasing concentrations of Ca²⁺ and Mg²⁺ from 0.25-4.0 mM were added before induction of ECP-release. Results are mean values of the number of experiments indicated in brackets, and the bars show S.E.M. The asterisk denotes p < 0.05 (Student's t-test).

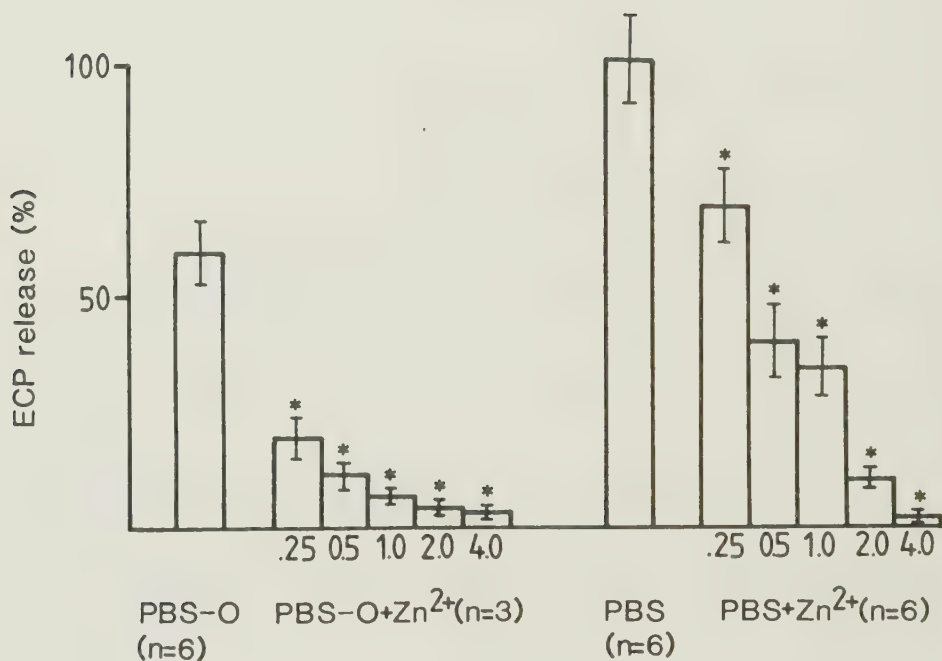


Fig. 2. Effects of Zn^{2+} on the release of ECP from eosinophils. The results obtained with PBS containing 2 mM Ca^{2+} and 2 mM Mg^{2+} were normalized to 100% (PBS). PBS-O is PBS without Ca^{2+} and Mg^{2+} . Zn^{2+} was added to the eosinophils in the concentrations shown at bottom of the figure. Results are mean values of the number of experiments shown within brackets and the bars show S.E.M. The asterisk denotes $p < 0.05$ (Student's t-test).

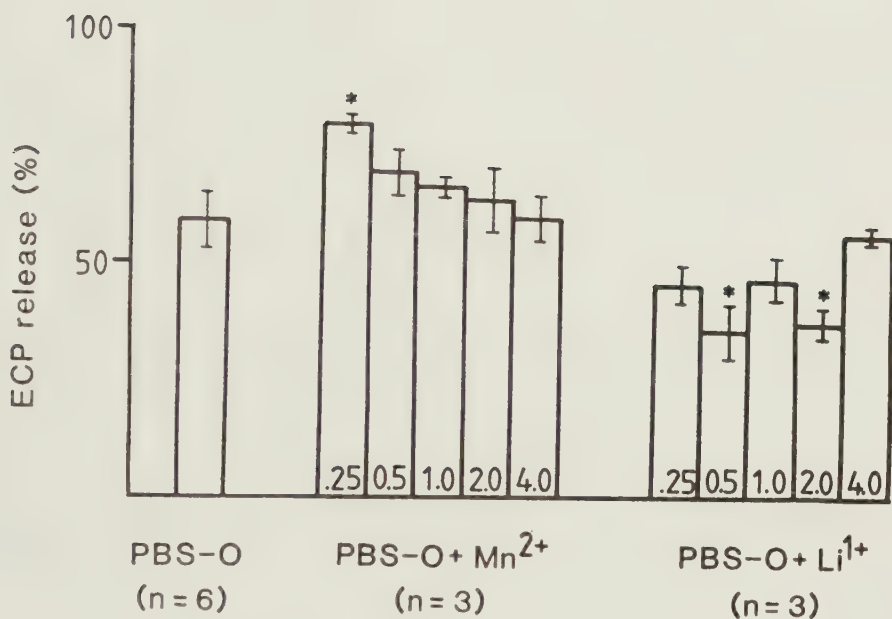


Fig. 3. Effects of Mn^{2+} and Li^{1+} on the release of ECP from eosinophils. ECP-release obtained with PBS with 2 mM Ca^{2+} and 2 mM Mg^{2+} was normalized to 100% and the other results were related to this value. PBS-O is PBS without Ca^{2+} and Mg^{2+} . Mn^{2+} and Li^{1+} was added to the eosinophils at the concentrations shown at bottom of the figure. Results are mean values $\pm$ S.E.M. and the number of experiments are shown within brackets. The asterisk denotes $p < 0.05$ (Student's t-test).

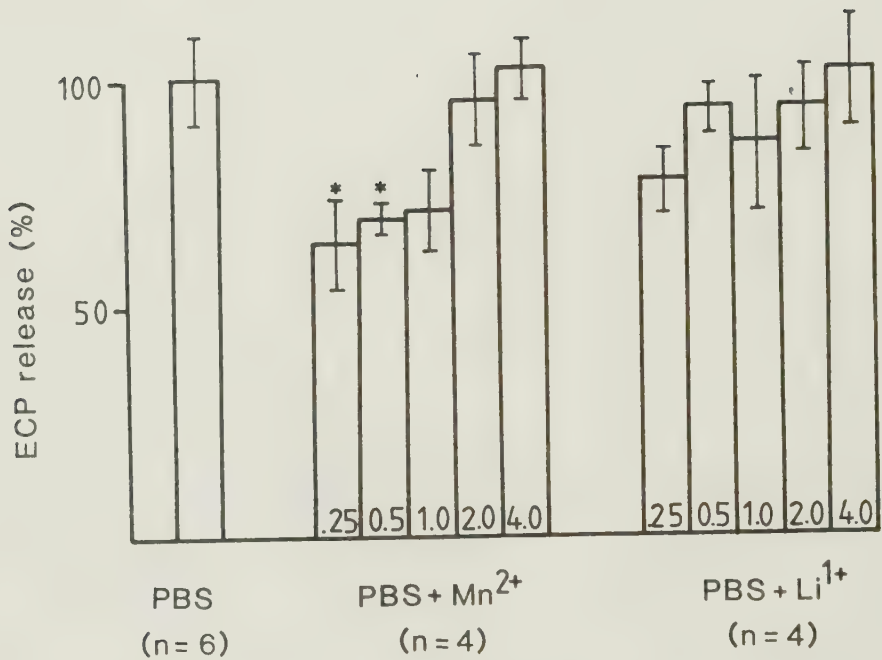


Fig. 4. Effects of Mn^{2+} and Li^{1+} on the release of ECP from eosinophils. ECP-release obtained with PBS with 2 mM Ca^{2+} and 2 mM Mg^{2+} was normalized to 100% and the other results were related to this value. Mn^{2+} and Li^{1+} was added to the eosinophils at the concentrations shown at bottom of the figure. Results are mean values $\pm$ S.E.M. and the number of experiments are shown within brackets. The asterisk denotes $p < 0.05$ (Student's t-test).

MECHANISMS FOR ADHERENCE OF EOSINOPHILS TO AN ANTIBODY COATED SURFACE

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SUMMARY

Eosinophils may act by degranulation after attachment to a surface. As the mechanisms of adherence are not understood, we have investigated the dependency on Fc(IgG) receptors and other mechanisms by studying the adherence of human eosinophils to albumin-Sepharose beads coated with either specific rabbit IgG antibody, F(ab')₂ antibody fragments or serum under different conditions. Adherence to Sephadex beads and albumin-coated microtitration plates was also investigated. Fifty percent of the eosinophils adhered spontaneously to all three different surfaces not coated with the antibody whereas only 25% of neutrophils and less than 10% of mononuclear cells adhered. A small but significant increase in adherence to albumin-Sepharose or albumin-coated plastic occurred after addition of the IgG-antibody, but not after addition of F(ab')₂ fragments, indicating that the Fc region was responsible for some increase in adherence. Incubation of eosinophils with IgG-Fc fragments prevented the additional antibody mediated adherence. As Fc-receptor negative eosinophils adhered almost as well as Fc-receptor positive cells, it appears that the Fc-receptors are of minor importance and instead, a nonspecific adherence mechanism, possibly unique for the eosinophil, seems to be the most important in eosinophil adherence to antibody coated surfaces.

Key words: eosinophil adherence - receptors for Fc(IgG).

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INTRODUCTION

Eosinophils may play a role in host resistance to helminthic infections by attachment and degranulation onto opsonized parasite surfaces. Mechanisms of adherence are therefore of importance. Less eosinophils than neutrophils form rosettes through Fc receptors with IgG-antibody coated erythrocytes (EA) (1-3) but in spite of this eosinophils have a greater capacity than neutrophils to kill IgG-antibody coated helminths in vitro (4). Also complement receptors as determined by rosette formation with complement coated sheep erythrocytes (EAC) are expressed to a lesser extent on eosinophils than neutrophils (1-2). Eosinophil EA rosetting is much more pronounced at 4° compared to 37° (5). However, this finding is not relevant for adherence of eosinophils to antibody-coated schistosomules since this is poor at 4° but markedly enhanced at 37° (5). Therefore it seems that adherence not mediated through Fc or complement receptors may also be of importance in eosinophil interaction with immunoglobulin coated parasites. It has been suggested that an initial temperature-independent adherence step is mediated through the Fc part of immunoglobulin while a second irreversible adherence step is temperature-dependent and perhaps related to degranulation (5).

To evaluate the mechanisms of adherence to an antibody coated surface, we studied the binding of eosinophils to Sepharose beads coated with immune complexes (albumin-antialbumin). Adherence to microtitration plates coated with immune complexes, and complement-coated Sephadex was also investigated.

MATERIALS

Hank's balanced salt solution (HBSS) and fetal-bovine serum (FBS) were from Flow Laboratories, England. Cytochalasin B from Calbiochem-Behring Corp., La Jolla, CA, was dissolved in dimethyl sulphoxide (DMSO) and diluted for use in HBSS to a final DMSO concentration of 0.5 %. Sephadex G15, Sephadex G200, CNBr-activated Sepharose, Percoll (polyvinylpyrrolidone-coated silica gel), Protein A - Sepharose CL-4B and Dextran 250 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Metrizamide and Lymphoprep (Isopaque-Ficoll) was from Nyegaard & Co, Oslo, Norway. Trypsin, human serum albumin (HSA) and Kabi-gamma were from Kabi, Stockholm, Sweden. 2-aminoethylisothiuronium bromide hydrobromide (AET), bovine serum albumin (BSA) and papain were from Sigma Chemical Corp., St Louis, MO. Pepsin was from Worthington Diagnostic System, Freehold, N.J. Varidase was from Lederle, Wayne, N.J. Gelatin was from Oxoid, Basingstoke, England.

Tyrode's solution; each liter of which contained 1 g glucose, 1 g NaHCO_3 , 0.2 g KCl, 8.0 g NaCl, and 0.05 g Na_2HPO_4 (anhydrous). Falcon 3040 96-well microtest II tissue culture plates (Tissue culture grade) were from Becton Dickinson Labware, Oxnard, CA. Polypropylen tubes were from Falcon Plastics, Los Angeles, CA.

Human serum was stored at -80° until used.

Isolation of eosinophils by density gradient centrifugation

Heparinized blood was collected from healthy volunteers with an eosinophil count $<0.4 \times 10^9/\text{l}$ blood and from volunteers with an eosinophilia (polyarteritis nodosa, lymphoma, filariasis, asthma). Five parts of blood were mixed with one part of 6% Dextran in 0.15 M NaCl and left at room temperature for 45 min for red cell sedimentation. The dextran-plasma was collected and centrifuged at 450 g for 5 min and the cells obtained were washed twice in HBSS containing 10 % heat-inactivated fetal-bovine serum (FBS) and Varidase (125 IE/ml). Washed leukocytes were centrifuged on a discontinuous metrizamide gradient according to Vadas et al. (6). A stock solution of 30 % (wt/vol) metrizamide was made in Tyrode's solution containing 0.1 % gelatin and 30 mg of Varidase per liter. Solutions were prepared in Tyrode's solution containing 18, 20, 22, 23, 24 and 25 % metrizamide and 1.8 ml aliquots were layered in 17x100 mm polypropylene tubes using a peristaltic pump (Minipuls II, Vilson, France). Two ml of cell suspension ($25-50 \times 10^6/\text{ml}$) in HBSS containing 10 % heat-inactivated FBS and Varidase (125 IE/ml) were layered on each gradient. The tubes were centrifuged at 1200xg for 45 min at room temperature. One ml fractions were collected from the bottom of the tubes and the cells were washed in HBSS. The eosinophil enriched fractions contained 82-94 % eosinophils. In some experiments neutrophils (more than 95%) and mononuclear cells (82-87% lymphocytes, 9-13% monocytes) were recovered from the metrizamide gradients or from Isopaque-Ficoll gradients respectively.

Cell surface receptors

Cell surface receptors for the Fc-part of IgG (FcR) and for complement were detected by use of sensitized sheep red blood cells (SRBC) in a rosetting assay. In brief, AET-treated (2-aminoethylsothiuronium bromide hydrobromide) (7) SRBC were sensitized as follows: 2% SRBC in PBS (PBS Dulbecco) containing 0.2 % BSA (PBSA) were mixed with an equal volume of rabbit anti-SRBC-IgG (dilution 1:32) and left at room temperature for 2 h, washed three times in PBSA and resuspended to 1% SRBC and used as IgG-coated SRBC(EA); alternatively 2% SRBC was mixed with an equal volume of rabbit anti-SRBC-IgM (dilution 1:32) at 37°C for 15 min, washed three times in veronal-buffered saline containing

2.5 mM Mg^{2+} and 0.75 mM Ca^{2+} and then incubated with an optimal amount of fresh mouse serum (strain A/Sn), final dilution 1:10 for another 15 min at 37° . After three washes in PBSA the cells were resuspended to 1% SRBC and used as C-coated SRBC(EAC).

Rosette-formation was induced by mixing 150 μ l of isolated eosinophils (2×10^6 /ml) with 150 μ l sensitized SRBC. After mixing the cells were chilled, pelleted by centrifugation at 600 r.p.m. and incubated in a refrigerator for 1 h. The cells were gently resuspended, toluidine blue dye solution was added and the number of rosetting cells counted in the microscope. 200-300 cells were counted. Each test contained control incubations with isolated cells and unsensitized SRBC. These controls were consistently negative.

Antibodies to human albumin

Rabbits were immunized with human serum albumin (HSA) and the antiserum used for preparation of $F(ab')_2$ fragments. Rabbit anti-HSA-IgG was prepared with caproic acid precipitation (8). The IgG fraction was dialyzed against 0.1 M sodium acetate pH 4.5 and pepsin (Worthington, 3000 U/mg) was added at 1 mg/50 mg IgG; pepsin-digestion occurred for 17 h at 37° and the solution was then applied to a 2.5×90 cm column of Sephadex G-200 in 0.03 M phosphate buffer pH 7.2 in 0.12 M NaCl. Fractions containing $F(ab')_2$ fragments and intact IgG were pooled and concentrated before separation on a protein A-Sepharose column (0.5×2 cm) eluted with the same buffer as used for dialysis. Fractions containing $F(ab')_2$ fragment, passing unabsorbed through the column, were pooled and used for absorption to HSA-coated Sepharose particles.

Human Fc-fragments were prepared from pooled gammaglobulin (Kabi-gamma) as follows: 1 ml containing 165 mg gammaglobulin was diluted to 8 ml with 0.01 M phosphate buffer pH 6.0 containing 0.001 M cysteine and 0.002 M EDTA, and heated to $37^{\circ}C$. Papain was added to a final concentration of 2 mg/ml and digestion was allowed for 5 h and discontinued by addition of 50 μ l 2.0 M Tris-HCl pH 8.0 per ml solution, immediately followed by 50 μ l/ml of iodoacetic acid (41 mg iodoacetic acid in 10 ml 2 M Tris-HCl pH 8.0). After dialysis against 0.03 M phosphate buffer pH 7.2 in 0.12 M NaCl the digested gammaglobulin was separated on G-200 as described. The broad lower molecular size peak containing Fc and $F(ab')$ fragments was collected, concentrated and run on protein A-Sepharose as described; the Fc fragments adsorbed to the column were eluted with 0.1 M glycine pH 3.0 and dialysed against PBS before use.

Separation of FcR(+) and FcR(-) eosinophils

In two experiments isolated eosinophils were separated into FcR(+) and FcR(-) cells for measurement of the adherence to albumin-coated Sepharose beads. Eosinophils, purity 95% and 88%, were rosetted with IgG-coated SRBC and separated in isosmolar Percoll gradients, density 1.091 g/ml (9) by centrifugation at $600 \times g$ for 20 min. The pellet contained more than 95% rosetting cells, and the cells from the interphase were more than 97% non-rosetting. FcR(-) cells were washed in HBSS before use in the adherence assay.

Trypsinization of cells

The eosinophils were incubated with 0.1 % trypsin at 37° for 30 min and washed with HBSS.

Adherence assay

Sepharose beads coated with albumin and antialbumin IgG and F(ab')₂

Human serum albumin was coupled to CNBr-activated Sepharose (albumin-Sepharose, AS) according to the manufacturers instructions. An aliquot of 800 μ l AS (=400 μ l packed AS) was incubated with 100 μ l of anti-albumin IgG (10 mg/ml) or antialbumin F(ab')₂ (2mg/ml) (ASAA). After incubation at 37° for 2 h ASAA was washed three times in HBSS and suspended to a final volume of 1000 μ l. A mixture of 400 μ l ASAA and 150 μ l eosinophils ($= 0.3 \times 10^6$) was incubated at 37° for 15 min and passed through a one-ml syringe with a nylon net at the bottom. Non adhered eosinophils were counted in the effluent. In some experiments non-adhered effluent eosinophils, collected in the effluent, were reincubated with ASAA and passed through the syringe a second time to determine the extent of nonspecific trapping in the Sepharose.

For comparison with albumin-coated Sepharose, CNBr-activated Sepharose was inactivated (IS), without coupling of a ligand, by treatment with 0.1 M Tris-HCl, pH 8 for 60 min.

Microtitration plates coated with albumin and antialbumin -IgG or antialbumin-F(ab)₂

Each well was treated with 300 μ l 0.1% HSA at room temperature for 24 h, washed three times with 0.15 M NaCl after which 300 μ l antialbumin IgG (10 μ g/ml) or antialbumin F(ab')₂ (40 μ g/ml) was added and the plates placed at 20° for 2 hours. After washing three times with PBS 200 μ l of an eosinophil suspension (1.0×10^6 /ml) was added and incubated for 15 min, after which the number of eosinophils remaining in suspension was determined.

Sephadex beads coated with serum

One ml of Sephadex G15 was incubated with normal serum or HBSS for 10 min at 37° and packed in a 2 ml syringe. Aliquots of 0.4 ml of cell suspension with 5×10^6 eosinophils per ml were drained into the columns and incubated for 15 min at 37° after which effluents were collected and cell counts performed.

RESULTS

EA and EAC rosette formation

The ability of eosinophils to form EA rosettes was determined in twelve healthy individuals and eleven patients with eosinophilia. EAC rosetting was determined in three healthy individuals and eleven patients with eosinophilia (polyarteritis nodosa, lymphoma, filariasis, asthma). The results demonstrate that the EA and EAC rosetting was similar for eosinophils obtained from both groups of individuals (Table I).

Non-specific and FcR dependent adherence to Sepharose

Isolated eosinophils, neutrophils and mononuclear cells were compared with respect to their adherence to albumin-Sepharose (Table 2); 56.1% of the eosinophils adhered whereas only 27.7% and 7.2% of the neutrophils and mononuclear cells respectively. When the albumin-Sepharose was coated with anti-albumin-IgG the fraction of adherent cells increased to 69.0% for eosinophils ($p < 0.01$) and 66.3% for neutrophils ($p < 0.05$). This increase is most likely due to adherence through receptors on the eosinophils and neutrophils for the Fc part of immunoglobulin. In line with this, treatment of albumin-Sepharose with $F(ab')_2$ fragments of anti-albumin did not result in an increased adherence, 61.1%, indicating that the Fc region of IgG is responsible for the increase in adherence. Furthermore, saturation of the Fc receptors on eosinophils by incubation with the isolated Fc fragments of human gammaglobulin (0.3 mg/ml for 10 min at 37°) abolished antibody mediated adherence indicating that no Fc receptors were available for binding of the eosinophil to anti-albumin coated Sepharose. Eosinophils adhered equally well to inactivated Sepharose (58.9%) as to albumin-Sepharose (56.1%).

Comparison of Sepharose with Sephadex - effects of serum

Eosinophils adhered to untreated Sephadex to a similar extent as to albumin-Sepharose, 52.6% and 56.1% respectively (Figure 1). However, treatment of albumin-Sepharose with fresh human serum resulted in a reduced adherence of eosinophils ($p < 0.01$), whereas serum treatment of Sephadex beads, presumably

leading to complement activation and coating of the beads with complement, increased the fraction of adherent cells from 50 to 95% ($p < 0.001$). On the other hand, heat-inactivated serum did not alter the adherence to either albumin-Sepharose or Sephadex beads (Figure 1).

The fraction of eosinophils that adhered to anti-albumin treated albumin-Sepharose (ASAA) did not change after pretreatment of ASAA with either fresh or heat-inactivated serum (Figure 2).

Non-specific and FcR dependent adherence to coated plastic surfaces

As a comparison with albumin-Sepharose we also investigated the adherence of eosinophils and neutrophils to an albumin-coated plastic surface, i.e. the bottom of microtiter wells. The results are shown in Table 3. Also to this surface almost twice as many eosinophils than neutrophils, 51.3% vs 28.3%, adhered during a 15 min incubation. When the albumin-coated plates were treated with anti-albumin-IgG the fraction of adherent cells increased significantly to 61.7% and 55.2% for eosinophils and neutrophils respectively. This increase in adherence is probably mediated over the Fc receptors on the cells binding to the Fc part of the anti-albumin. This notion is sustained by the observation that when the $F(ab')_2$ fraction of anti-albumin was used instead of intact IgG there was no increased adherence (Table 3).

Adherence of recycled eosinophils

When eosinophils, which did not adhere to AS, were incubated once more with AS, a fraction of 63% adhered compared to 72% during the first cycle. When eosinophils, which had not adhered to ASAA the first time, were incubated once more with ASAA, 57% of the cells adhered compared to 76% during the first cycle (Table 4). Thus the adherence is mostly a random phenomenon since a large fraction adhered also during a second cycle.

Adherence of Fc receptor negative cells

FcR-negative eosinophils were isolated from one normal subject and one patient with eosinophilia (eosinophilic pulmonary infiltration); 61% and 50% respectively of the FcR-negative cells adhered to AS, and 67% and 60% to ASAA. These results sustain the notion that most of the adherence even to ASAA is a FcR independent phenomenon.

Effect of temperature and Cytochalasin B on eosinophil adherence

The adherence of eosinophils to ASAA was lower at 4°C as compared to a control incubation at 37°C (Fig 3). Also treatment with Cytochalasin B (10 µg/ml) decreased the adherence (Fig 3). It is, however, not possible to decide whether Fc mediated or unspecific adherence was inhibited.

Effect of trypsin on eosinophil adherence

Treatment of eosinophils with trypsin did not change the adherence of eosinophils to AS or ASAA (Table 5).

DISCUSSION

Eosinophils may act by adherence to and degranulation onto noningestible surfaces. Sepharose (10) and Sephadex (11) treated with serum have previously been shown to provide surfaces suitable for induction of release of granule proteins, but the mechanisms for adherence of the eosinophils are not fully understood. It is a reasonable assumption that specific binding mediated by Fc(IgG)-receptors or complement-receptors may play a role. However, the finding that approximately 50% of the eosinophils adhered to albumin-Sepharose, albumin-coated plastic surfaces or untreated Sephadex suggested that nonspecific binding (i.e. other than FcR or complement-receptor mediated) also may be important. The observation that only 25-30% of isolated neutrophils adhered to albumin-Sepharose or albumin-coated plastic surfaces, and that only 7% of mononuclear blood cells adhered to albumin-Sepharose, strongly suggests that the high non-specific binding of eosinophils to these surfaces is a unique property of the eosinophils.

The increase in eosinophil adherence to albumin-Sepharose or to albumin-coated microtiter wells seen after addition of antialbumin indicates some Fc-receptor dependent binding. In line with this F(ab')₂ fragments did not increase the adherence, and furthermore, precubination of the eosinophils with Fc fragments abolished the presumed antibody-mediated adherence. The increase is not impressive which probably is a reflection of the fact that only 20-25% of the eosinophils have Fc-receptors. As a comparison, the neutrophils used in these studies showed a doubling in adherence after addition of antialbumin corresponding to their higher frequency of Fc-receptors (75-80% using the same rosetting assay as for eosinophils).

It can be argued that the Fc-receptors may be involved also in the nonspecific binding to albumin-Sepharose or albumin-coated plastic surfaces. However, the fraction of adhering cells is much greater than the fraction of cells bearing Fc-receptors, and the finding that also isolated Fc-receptor negative cells adhered to the same extent as a mixture of Fc-receptor positive and negative eosinophils suggests that most if not all of the nonspecific binding of eosinophils is independent of the Fc-receptors. This interpretation is also supported by the fact that a fraction of eosinophils which did not adhere during a first adherence cycle still had the capacity to adhere during a second cycle. Therefore, the Fc-receptor dependency does not seem to be the most important factor for eosinophil adherence to an antibody coated surface.

The percentage of eosinophils forming rosettes with IgG-coated erythrocytes varies depending on the species in which the antibody is produced (1). We used rabbit antibody and the percentage of rosettes formed with sheep erythrocytes coated with antibody was as previously reported (2). Others have found that eosinophils from patients with helminthic infections express more Fc-receptors than eosinophils from healthy individuals (12). In another study, no difference in Fc-receptors was found between eosinophils from healthy individuals and patients with eosinophilia (2). Most of the patients in the latter study, however, had allergic diseases such as rhinitis, asthma and drug hypersensitivity and not helminthic infections. Similarly we did not find any difference in Fc-receptors between eosinophils from healthy individuals and patients with eosinophilia, except for one patient with filariasis who had an increased EA rosetting of eosinophils.

Like Fc-receptors, complement receptors are expressed to a lower extent on eosinophils than neutrophils, but the difference is less marked. The possible importance of the eosinophil complement receptors has been demonstrated in studies of eosinophil degranulation upon adherence to serum-treated Sepharose (10), and serum-treated Sephadex (11). Sephadex treated with fresh serum but not heat-inactivated serum was actually the most efficient surface for eosinophil adherence in the present study indicating a role of a heat-labile factor for adherence.

The initial adherence of eosinophils to antibody-coated schistosomules is suggested to be a temperature independent interaction via Fc-receptors and a second step that is temperature dependent and related to degranulation (5). We found that the adherence of eosinophils to ASAA was lower at 4°C compared to 37°C, but it is not possible to decide whether it is Fc-mediated or nonspe-

cific adherence that is decreased at 4°C. Cytochalasin B is reported to reduce the fraction of FcR positive cells (13). Pretreatment of the cells with Cytochalasin B, like low temperature, inhibited the adherence. Again it is, however, not possible to decide what kind of adherence is inhibited, Fc-receptor mediated or nonspecific. The adherence to serum-treated Sephadex was not inhibited by Cytochalasin B indicating separate mechanisms for adherence to serum-treated Sephadex (mainly through C-receptors) and to ASAA (mainly nonspecific and through Fc-receptors).

Trypsin treatment of the eosinophils, which increases EA binding while destroying EAC binding (13) did not increase Fc-receptor dependent adherence. These results too suggest that the Fc-receptor dependency is not the most important factor for eosinophil adherence to ASAA. Degranulation is suggested to play a role for continued eosinophil adherence (5). The finding that serum-treated Sephadex can induce release of granule contents (11) and at the same time is most efficient in stimulating the eosinophils to adhere also suggest a role for granule contents in adherence.

Undoubtedly both Fc(IgG) and complement receptors are important for certain aspects of adherence to different surfaces but our findings clearly demonstrate that also other mechanisms play a role for surface adherence of eosinophils.

ACKNOWLEDGEMENTS

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TABLE 1.

Fc-receptors (EA-rosettes) and complement receptors (EAC-rosettes) on eosinophils.

	EA-rosettes(%)	EAC-rosettes(%)
Normals (n=12)	20.8 $\pm$ 3.1	-
" (n=3)	-	60.7 $\pm$ 10.0
Patients with		
eosinophilia (n=11)	26.0 $\pm$ 3.6	66.6 $\pm$ 4.8

Results are shown as mean $\pm$ S.E.M. There are no statistical differences between normals and patients with an eosinophilia.

TABLE 2.

Adherence of eosinophils, neutrophils and mononuclear cells to Sepharose after various treatments.

Surface	Adherent cells (%)		
	Eosinophils	Neutrophils	Mononuclear cells
Albumin-Sepharose (AS)	56.1 $\pm$ 3.7 (21)	27.7 $\pm$ 4.3 (3)	7.2 $\pm$ 4.9 (3)
AS+antialbumin-IgG (ASAA)	*69.0 $\pm$ 2.9 (21)	*66.3 $\pm$ 1.4 (3)	18.7 $\pm$ 7.5 (3)
AS+antialbumin-F(ab) ₂	61.1 $\pm$ 5.3 (8)	NT	NT
ASAA; cells preincubated with Fc-fragments	48.6 $\pm$ 8.9 (3)	NT	NT
Inactivated Sepharose (IS)	58.9 $\pm$ 1.0 (2)	NT	NT

The asterisk denotes a significant difference ($p < 0.05$) in the fraction of adherent cells when compared with the adherence to AS. NT, not tested. The numbers within brackets show the number of experiments. Mean values $\pm$ SEM are shown.

TABLE 3

Adherence of eosinophils and neutrophils to microtitration plates (P) after various pretreatments

Surface	Adherent cells (%)	
	Eosinophils	Neutrophils
Albumin-coated (AP)	51.3 $\pm$ 3.0 (5)	28.3 $\pm$ 3.3 (6)
AP+antialbumin-IgG (APAA)	*61.7 $\pm$ 3.3 (5)	*55.2 $\pm$ 5.2 (6)
AP+antialbumin-F(ab') ₂	43.2 $\pm$ 2.6 (5)	27.7 $\pm$ 5.3 (4)

The asterisk denotes a significant difference ($p < 0.05$) in the fraction of adherent cells compared with the adherence to AP. The numbers within brackets show the number of experiments. Mean values $\pm$ SEM are shown.

TABLE 4.

Adherence of recycled eosinophils.

Surface	Adherent cells(%) 1st incubation	Adherent cells(%) 2nd incubation
Albumin-Sepharose with anti-albumin(ASAA) (n=6)	75.6 $\pm$ 3.4	56.6 $\pm$ 8.2
Albumin-Sepharose(AS) (n=4)	71.9 $\pm$ 4.9	62.5 $\pm$ 1.4

Eosinophils were passed through ASAA and AS respectively; the fraction of adherent cells are shown in the left column (mean $\pm$ S.E.M.). Non-adherent cells were recycled through fresh columns of ASAA and AS; the fraction of adherent recycled cells is shown in the right column.

TABLE 5.

Adherence of eosinophils after trypsinization.

Surface	Adherent cells(%) - control	Adherent cells(%) - trypsinized	
Albumin-Sepharose(AS) (n=6)	55.1 $\pm$ 6.6	60.8 $\pm$ 5.2	p > 0.1
Albumin-Sepharose + anti-albumin(IgG) (ASAA) (n=8)	68.7 $\pm$ 3.4	73.8 $\pm$ 4.1	p > 0.1
Albumin-Sepharose + anti-albumin(F(ab') ₂) (n=4)	69.5 $\pm$ 4.3	73.3 $\pm$ 3.3	p > 0.05

Eosinophils were treated with trypsin, 0.1%, at 37°C for 30 min and then washed before the adherence assay. Results are mean $\pm$ S.E.M. and p-values are from Student's t-test.

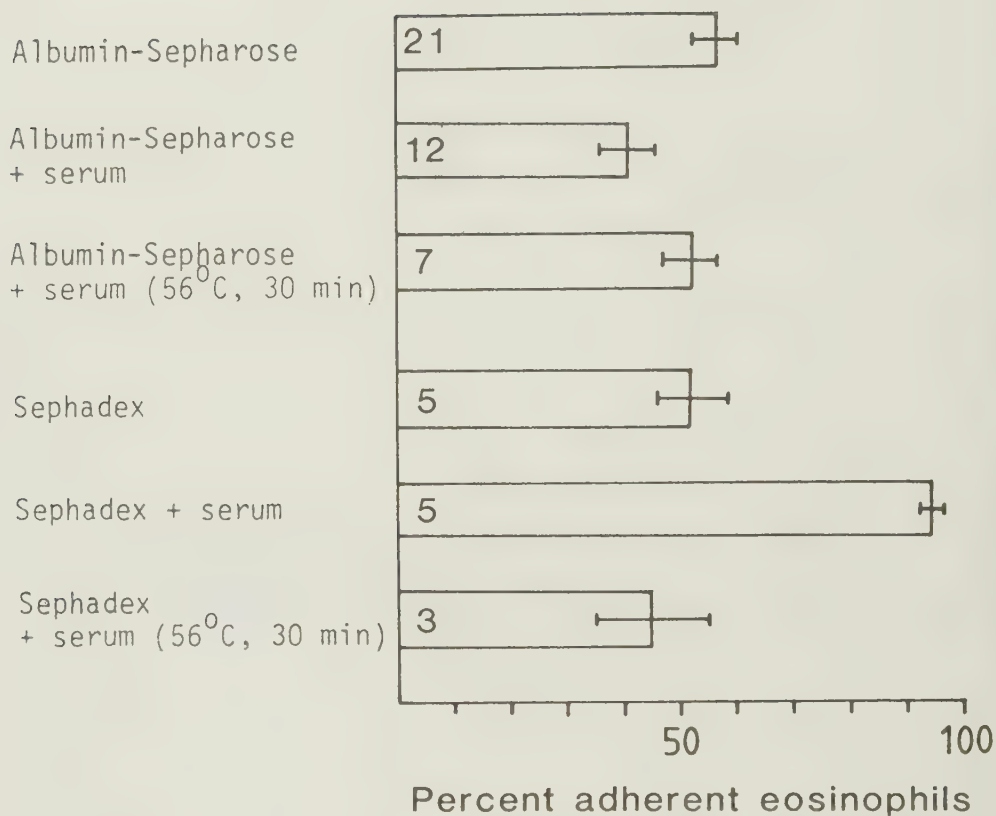


Fig. 1. Adherence of eosinophils to albumin-Sepharose and Sephadex. Albumin-Sepharose and Sephadex were treated with fresh or heat-inactivated serum for 15 min and washed three times before incubation with eosinophils for 15 min. Additional experiments with inactivated Sepharose (IS) showed the same results as with AS. Untreated beads were run as controls. The number of experiments are shown within each column and bars indicate SEM.

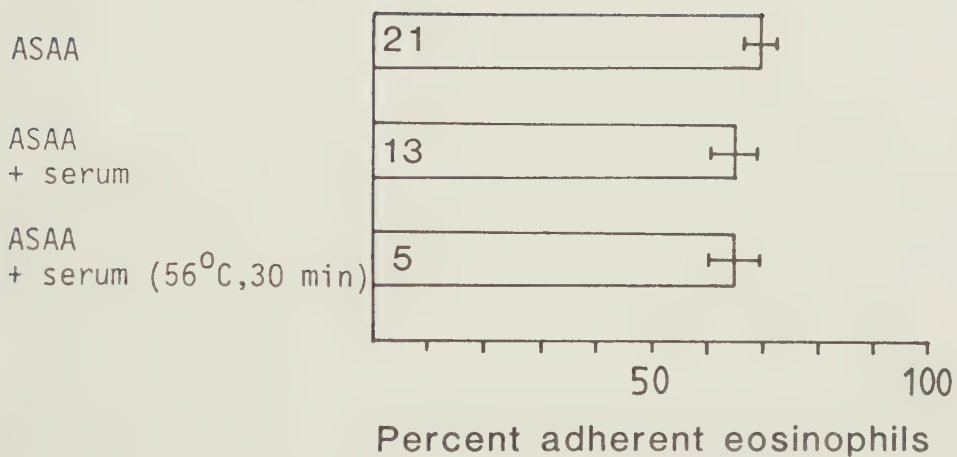


Fig. 2. Adherence of eosinophils to ASAA (albumin-Sepharose coated with antialbumin IgG). ASAA was treated with fresh or heat-inactivated serum for 15 min and washed three times before incubation with eosinophils for 15 min. The number of experiments are shown within each column and bars indicate SEM.

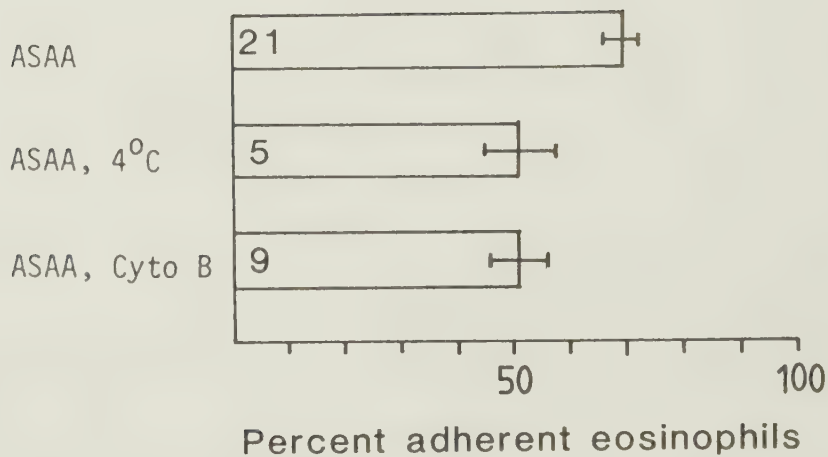


Fig. 3. Adherence of eosinophils to ASAA (albumin-Sepharose coated with antialbumin IgG) at 4°C and after preincubation of the cells with Cytochalasin B (10 µg/ml). The number of experiments are shown within each column and bars indicate SEM.

Altered density, metabolism and surface receptors of eosinophils in eosinophilia

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Summary. A comparison was made between eosinophils from normal persons and patients with eosinophilia. Highly purified eosinophils were obtained by centrifugation in a Percoll density gradient. Studies were carried out on density distribution, oxygen consumption upon adherence to serum-coated Sephadex and expression of cell surface receptors for IgG and complement. In eosinophil leukaemia the density of eosinophils was abnormally low. Abnormal light density fractions of blood eosinophils were also detected in the hypereosinophilic syndrome (HES). Light density eosinophils of HES showed morphological signs of degranulation consonant with the finding of a low content of granular eosinophil cationic protein (ECP) suggesting degranulation in the circulation or abnormal granule formation in the marrow. In addition, such cells exhibited a higher oxygen consumption than eosinophils with normal density upon adherence to serum-coated Sephadex. Low density eosinophils showed a greater number of cells with Fc-IgG and complement receptors than high density cells. Likewise exudate eosinophils displayed an abnormally low density with higher than normal oxygen consumption indicating that eosinophils may be activated in the tissues. In one patient with HES, a febrile episode resulted in a disappearance of eosinophils with a normal density while abnormal low density eosinophils increased. Our findings suggest

that eosinophils from some patients with eosinophilia may be 'activated' in the circulation.

INTRODUCTION

Eosinophilia is typically associated with allergic and parasitic disease but it can also be a feature of other disorders such as immune complex diseases. The function of the eosinophil is a matter of controversy. One concept suggests a modulatory role in the anaphylactic reaction (Goetzel, Weller & Valone, 1979) and another a role in killing of parasites (Kay, 1979). Eosinophil granules contain unique proteins such as the major basic protein (MBP; Gleich, Loegering, Kueppers, Bajaj & Mann, 1974; Gleich, Loegering, Mann & Maldonado, 1976; Gleich, Loegering & Maldonado, 1973) and the eosinophil cationic protein (ECP; Olsson & Venge, 1974; Olsson, Venge, Spitznagel & Lehrer, 1977). These proteins were toxic to schistosomules of *Schistosoma mansoni* (Butterworth, Wassom, Gleich, Loegering & David, 1979; McLaren, McKean, Olsson, Venge & Kay) and to a variety of mammalian cells (Gleich, Frigas, Loegering, Wassom & Steinmüller, 1979; Frigas, Loegering & Gleich, 1981). Extracellular release of such agents with damage to tissues may therefore be suggested as responsible for cardiovascular complications of the hypereosinophilic syndrome (Spry, 1980). Eosinophil production of oxygen products including superoxide anion, hydroxyl radical, and hydrogen peroxide might also lead to tissue damage. In certain states of high

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eosinophilia, eosinophils could be activated both to release granular cationic proteins and produce active oxygen products. Morphological abnormalities including vacuolated and degranulated eosinophils as well as increased rosette formation with antibody-coated erythrocytes (Tai & Spry, 1976) support this view. Furthermore eosinophils from patients with eosinophilia appeared activated when compared with normal eosinophils by surface charge, activation of acid phosphatase, membrane hexose transport, and hexose monophosphate shunt activities (Bass, Grover, Lewis, Szedja, de Chatelet & McCall, 1980).

The present work was undertaken to compare eosinophils from normal persons with those from patients with eosinophilia. We performed studies on density distribution, oxygen consumption upon adherence to serum-coated Sephadex (Metcalf, Gadek, Raphael, Frank, Kaplan & Kaliner, 1977) and expression of cell surface receptors for IgG and complement. Our results indicate that 'activated' eosinophils circulate in certain hypereosinophilic states.

MATERIALS AND METHODS

The patient material is presented in Table 1. No treatment had been given to these patients.

Isolation of eosinophils by centrifugation in a Percoll density gradient

Heparinized blood was collected from healthy volunteers (eosinophils $< 400/\mu\text{l}$) and from volunteers with eosinophilia (Table 1). Five parts blood were mixed

with one part 6% Dextran (Dextran 250, Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.15 M NaCl and left at room temperature for 45 min to allow the red cells to sediment. The dextran-plasma was collected and centrifuged at 450 *g* for 5 min. The cells were washed twice in Hanks's balanced salt solution (HBSS; Flow Laboratories).

A density gradient of Percoll (polyvinylpyrrolidone-coated silica gel, Pharmacia Fine Chemicals, Uppsala, Sweden) was made using a modification of Gärtnert (1980). Percoll and HBSS were mixed to obtain solutions of different densities at pH 7.4. The osmolarity was adjusted with either HBSS or water to 277 mosm. Gradients were formed using a peristaltic pump (Minipuls II Gilson, France) and consisted of Percoll solutions with the following densities (g/ml): 1.100 (1 ml), 1.095 (3 ml), 1.090 (3 ml) and 1.085 (3 ml) layered on top of each other in 17 × 100 mm polypropylene tubes (Falcon Plastics, Los Angeles, Calif.). After washing, the cells were suspended in Percoll (1.075 g/ml) and adjusted to $25 \times 10^6/\text{ml}$. Two millilitre of the cell suspension were layered on each gradient followed by centrifugation in an angle rotor (angle 45°) at 1600 *g* for 20 min at room temperature. One millilitre fractions were collected from the bottom of the tubes using a peristaltic pump. The density of each fraction was measured and the cells were washed in HBSS and counted. Cytocentrifuge smears were prepared for differential counts.

Assay for eosinophil cationic protein

Isolated granulocytes were extracted with 0.3% cetyltrimethyl ammonium bromide (CTAB, BDH, Poole) in 0.01 M sodium phosphate buffer pH 7.0. One millilitre

Table 1.

Patients	Age	Sex	WBC $\times 10^9/\text{litre}$	Eosinophils (%)
A. Hypereosinophilic syndrome	48	M	20.3	45.3
Hypereosinophilic syndrome	36	F	28.9	57.4
Eosinophil leukaemia	25	M	11.5	47.0
Sideroblastic anaemia + eosinophilia	69	M	11.9	61.5
B. Polymyalgia rheumatica	83	M	6.6	13.5
Rheumatoid arthritis	63	F	12.6	39.7
C. Filariasis	34	M	70.0	85.5
Anchylostomiasis	29	M	9.1	36.0
D. Hodgkin's lymphoma	57	M	15.4	57.7
Primary hepatocellular carcinoma	54	F	40.8	9.0
Eosinophil gastroenteritis	64	F	6.5	41.0

of this solution was added per 10^7 cells and extraction was carried out at 0° with frequent mixing using a pasteur pipette. ECP was quantified with the single radial immunodiffusion method of Mancini, Carbonara & Heremans (1965) using an antiserum against ECP (Venge, Roxin & Olsson, 1977a). Purified ECP (Olsson *et al.*, 1977) was used as reference standard.

Oxygen consumption

Oxygen consumption was stimulated with serum-treated Sephadex beads (Pharmacia, Uppsala, Sweden). The Sephadex beads were suspended in HBSS (0.3×10^6 /ml). This suspension (0.8 ml) was incubated with 0.7 ml normal human serum (stored at -80°) at 37° for 10 min and mixed with 1 ml cell suspension to a final concentration of 4×10^6 cells/ml (purity 82%–97%). Thereafter oxygen consumption was measured with a Clark electrode mounted in a 2.24 ml glass-stoppered incubation chamber equipped with magnetic stirring and heating to 37° (Eschweiler & Co, Kiel, Germany). A paper recorder was connected for continuous recording of the oxygen tension. The electrode was calibrated with pure N_2 and 12.25% oxygen in N_2 . Oxygen consumption, expressed as $\mu l O_2/10^7$ granulocytes/min, was calculated according to the following formula:

$$\text{ml } O_2 = pO_2 \times \frac{\alpha}{760} \times V$$

where α is the absorption coefficient (0.02350) and V is the volume of the reaction chamber (2.24 ml).

Cell surface receptors

Fc receptors for IgG were determined by a rosette assay using ox erythrocytes coated with rabbit antibodies (Hallberg, Gurner & Coombs, 1973). Complement receptors were determined by a rosette assay using sheep erythrocytes coated with mouse complement. For this test sheep erythrocytes coated with a small dose of rabbit IgM antibodies were incubated with an optimal amount of fresh mouse serum (strain A/SN) for 15 min at 37° . After repeated washes the cells were made up to 1% in Dulbecco's saline with 0.2% bovine serum albumin. One hundred microlitres of cells (2×10^6 /ml) and 100 μl of indicator red cells were mixed in a 3 ml plastic tube and rotated for 1 hr at 37° . Aliquots of the cell suspensions were then mixed with toluidine blue dye and counted in a microscope as for the Fc rosette assay. Each test contained controls with untreated sheep erythrocytes as well as sheep erythrocytes coated with rabbit antibodies but lacking

mouse complement. These controls were consistently negative.

RESULTS

Density distribution of eosinophils and relationship to content of ECP

Figure 1 shows the density distribution in Percoll of eosinophils from healthy individuals. The recovery of eosinophils was 42%–62%. The peak density is 1.088 g/ml. Neutrophils were found to have a lower density with a peak at 1.081 g/ml.

An identical distribution was found in the density gradient of eosinophils and immunochemically determined ECP (data not shown) both in healthy individuals and patients with eosinophilia, but not in eosinophil leukaemia. These findings would be expected if ECP is a unique constituent of eosinophils. The relationship between density of eosinophils and content of ECP was also analysed (Fig. 2).

Eosinophils with a low density had a lower content of ECP than those with a normal density. Therefore the ECP content is a parameter of cell density, which most likely is a function of the number and properties of eosinophil granules. However, in eosinophil leukaemia the content of ECP was very low and the

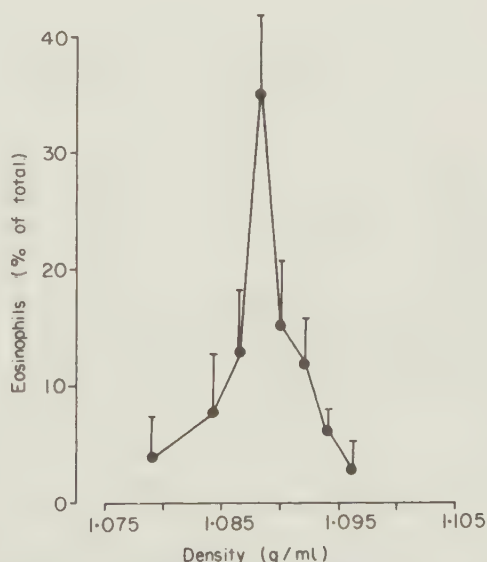


Figure 1. Density distribution of eosinophils from healthy individuals in a Percoll gradient ($n = 5$). Eosinophils peak at a density of 1.088 g/ml.

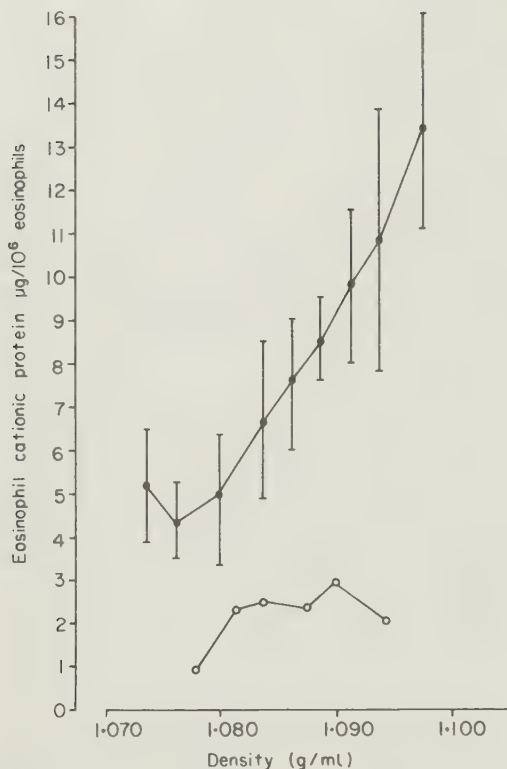


Figure 2. Relationship between density of eosinophils and content of ECP. Cells from nine patients with eosinophilia were centrifuged in Percoll. Eosinophils were recovered and assayed for ECP content (●—●). There is a decreasing content of ECP with decreasing cell density. Data on one patient with eosinophil leukaemia are presented separately (○—○) as they show abnormally low ECP content.

relationship to cell density was less obvious compared to non-leukaemic eosinophilia.

Density distribution of eosinophils in eosinophilia

Figure 3 shows the density distribution of eosinophils from eleven patients with eosinophilia. Cells (50×10^6) were applied to each gradient. The number of eosinophils varied from 4.5×10^6 to 42.8×10^6 . The recovery of eosinophils was 77.8% (56.0–98.0). In eosinophil leukaemia an abnormally low density was found. In hypereosinophilic syndrome (HES) eosinophils had either a low density or showed populations with both normal or low density. Eosinophils from patients with immune complex diseases (rheumatoid arthritis and polymyalgia) and eosinophilia displayed a normal

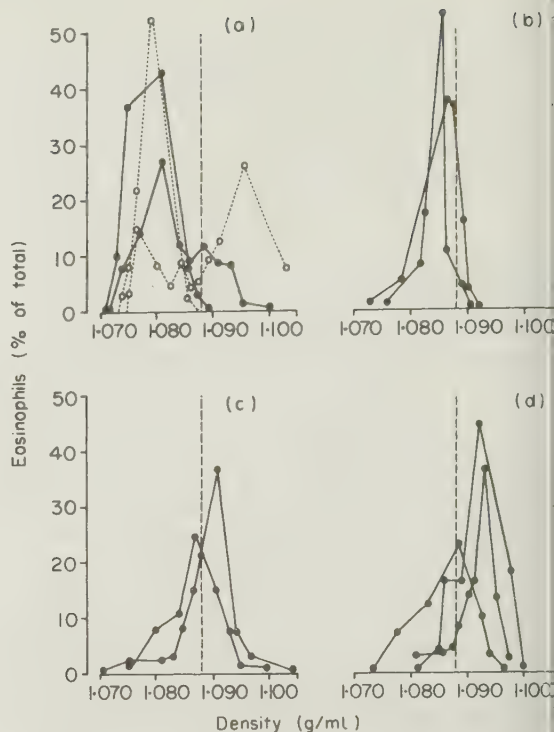


Figure 3. Density distribution of eosinophils from patients with eosinophilia. The broken vertical line indicates the peak density of normal eosinophils. Eosinophils from one patient with eosinophil leukaemia and one patient with sideroblastic anaemia, eosinophilia and abnormal karyotype (●—●) have abnormally low density while two patients with hyper-eosinophilic syndrome (○---○) show a more heterogeneous pattern (a). In two patients with immune complex disease (rheumatoid arthritis and polymyalgia rheumatica) eosinophils show a normal density (b). Eosinophils from patients with parasite infections (c), and eosinophil gastroenteritis, primary hepatocellular carcinoma and Hodgkin's lymphoma (d) also exhibit a normal density.

density. Eosinophils from patients with parasite infections, eosinophil gastroenteritis, primary hepatocellular carcinoma and lymphoma also exhibited a normal density.

Eosinophil density, oxygen consumption and cell surface receptors in the hypereosinophilic syndrome

Figure 4 depicts studies of eosinophils from a patient with HES exhibiting both abnormally 'light' and 'heavy' eosinophils. The data of Fig. 4 are given in relative numbers. However, also when the data are given in absolute numbers a clear shift in density of the

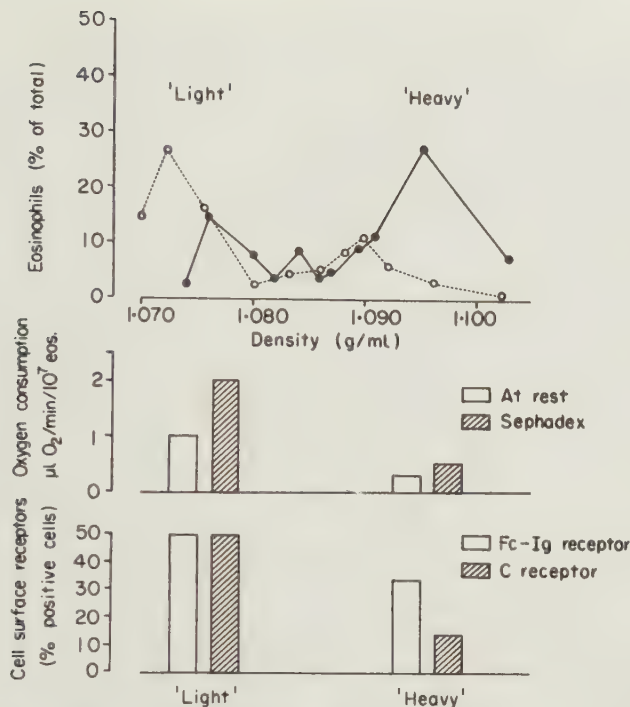


Figure 4. Studies of eosinophil density, oxygen consumption and cell surface receptors in a patient with hypereosinophilic syndrome (●—●); and during a febrile episode (○---○). Isolated 'light' and 'heavy' eosinophils were assayed for oxygen consumption at rest and after adherence to serum-treated Sephadex. Fc-IgG receptors and complement receptors were also determined.

eosinophils was seen during the febrile episode. Thus before the febrile episode $9.6 \times 10^9/l$ and $2.7 \times 10^9/l$ could be calculated to represent 'heavy' and 'light' eosinophils, respectively, per litre blood. During the febrile episode the numbers were $3.74 \times 10^9/l$ and $6.59 \times 10^9/l$ for 'heavy' and 'light' eosinophils, respectively. Striking differences were observed between light and heavy eosinophils (Fig. 4). Thus, oxygen consumption both at rest and upon adherence to serum-treated Sephadex was much higher for light than heavy eosinophils. Furthermore, 50% of light eosinophils showed Fc-IgG receptors and 50% showed complement receptors while heavy eosinophils displayed 34% and 14%, respectively.

Another HES patient with only abnormally light eosinophils (Fig. 3) showed 84% eosinophils with Fc-IgG receptors and 69% with complement receptors on their surface (data not shown).

In addition light eosinophils appeared degranulated as compared with heavy eosinophils (Fig. 5).

Comparisons between eosinophils from blood and pleural exudates

Comparative studies were done between blood and exudate eosinophils in one patient with eosinophilia (1.6×10^9 peripheral blood eosinophils/litre) and a pleural exudate with eosinophilia of unknown etiology (Fig. 6). Data for this patient are not included in Table 1. Blood eosinophils showed a normal density while pleural exudate eosinophils displayed an abnormally low density. Furthermore, pleural eosinophils showed a higher oxygen consumption than blood eosinophils (data not shown).

Oxygen consumption of eosinophils from patients with eosinophilia

These particular studies were carried out only with eosinophils from patients with eosinophilia because it was not possible to obtain enough eosinophils from normal persons. Figure 7 shows oxygen consumption

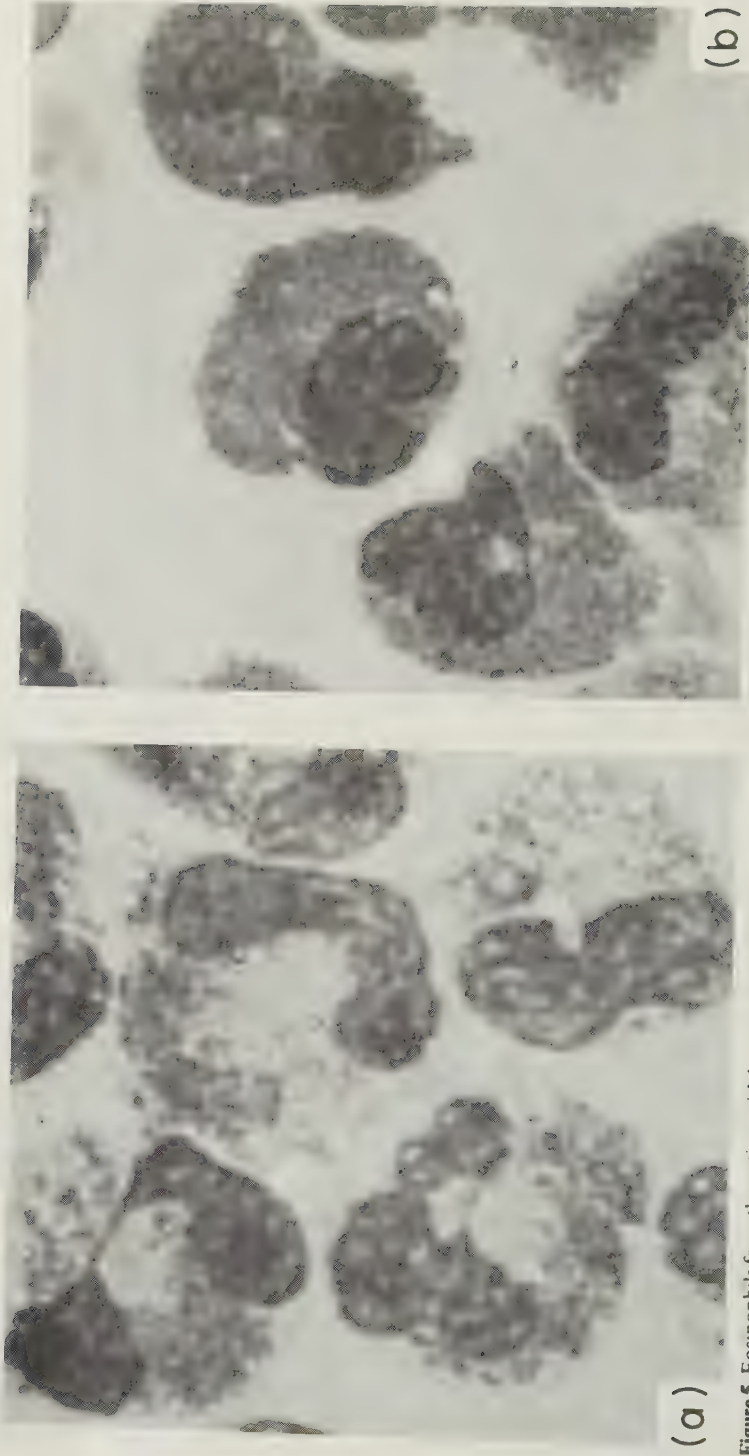


Figure 5. Eosinophils from the patient with hyper-eosinophilic syndrome described in Fig. 4 (a) 'Light', degranulated eosinophils, (b) 'Heavy' eosinophils with normal granulation.

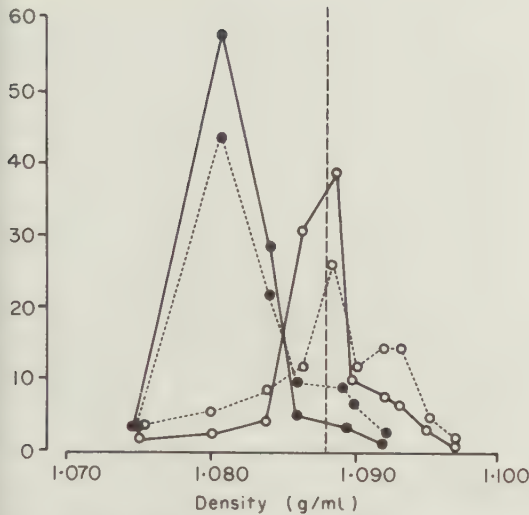


Figure 6. Comparisons of eosinophils isolated from blood and a pleural exudate in a patient with eosinophilia of unknown etiology. Density of blood eosinophils (○—○) and density of pleural eosinophils (●—●). Content of ECP of blood eosinophils (○---○) and pleural eosinophils (●---●).

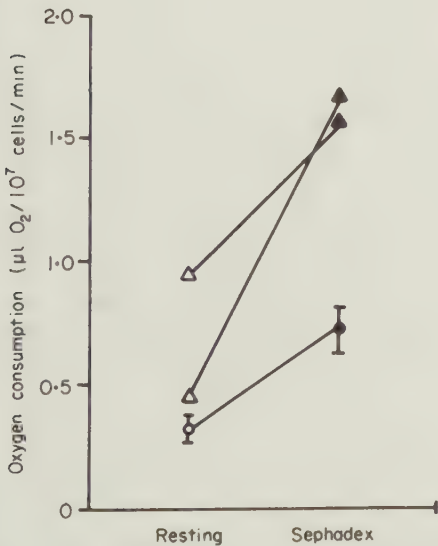


Figure 7. Oxygen consumption of eosinophils from patients with eosinophilia isolated by centrifugation in Percoll. 'Light' eosinophils from two patients with HES at rest (Δ) and during interaction with serum-treated Sephadex (▲). Eosinophils from six patients with eosinophilia (group b, c and d of Fig. 3) at rest (○) and during stimulation with serum-treated Sephadex (●).

for eosinophils at rest and during interaction with serum-treated Sephadex beads. The oxygen consumption usually increased two–three times upon contact with the opsonized beads. Light density eosinophils from two patients with HES showed a higher oxygen consumption upon contact with serum-treated Sephadex than eosinophils from other patients with eosinophilia or normal density eosinophils from patients with HES. Light eosinophils from one of these patients also exhibited an increase in oxygen consumption at rest (Figs 4 and 7).

Isolated neutrophils displayed a similar respiration as eosinophils at rest but only a minimal increase when added to the opsonized Sephadex beads (data not shown).

DISCUSSION

Our findings suggest that eosinophils from some patients with eosinophilia, especially those with the hypereosinophilic syndrome, may be 'activated' in the circulation. This is indicated by a decrease in cell density, increased expression of cell surface receptors, and increased oxygen consumption. It should, however, be emphasized that only a limited number of patients with this rare disorder could be investigated. Therefore, the present studies have to be repeated in a larger patient group before the clinical importance of the findings can be evaluated. Nevertheless, the present findings may offer explanations for the pathophysiology of HES.

The low density of eosinophils in HES can probably be explained by a partial degranulation in the circulation with release of granule contents. Alternatively, light density cells of HES may represent an abnormal (preleukaemic?) population which is produced in the marrow with a lower granule content than normal. But the correlation observed earlier between the number of morphologically appearing degranulated blood eosinophils and the serum level of the eosinophil cationic protein (ECP) determined by a radioimmunoassay (Venge *et al.*, 1977a) indicates that ECP is actually released in the circulation of patients with HES (Spry, 1980). Fluctuations of serum ECP have also been reported in patients with asthma (Venge, Zetterström, Dahl, Roxin & Olsson, 1977b; Dahl, Venge & Olsson, 1978) and other allergic diseases (Winqvist, Olsson, Werner & Stenstam, 1981). In the latter instances fluctuations of serum ECP may reflect release from circulating or tissue eosinophils although

changes in turnover rates also have to be considered. Furthermore, elevated concentrations of major basic protein of serum are present in some diseases associated with eosinophilia (Wassom, Loegering, Solley, Moore, Schooley, Fauci & Gleich, 1981). Thus, several observations suggest the possibility that eosinophils can be degranulated in certain disease states.

The abnormally low density of eosinophils from patients with eosinophil leukaemia indicating a low granule content or abnormal granule composition is probably a result of the malignant character of such cells rather than secondary degranulation. Thus, in contrast to findings in HES spontaneous and stimulated oxygen consumption were not elevated. Furthermore, the ECP content was extremely low in one patient with eosinophil leukaemia without a clear relationship to density of the cells, indicating abnormalities of granule composition. Otherwise we found a correlation between density distribution and ECP content of eosinophils from both healthy individuals and patients with eosinophilia strongly indicating that ECP is actually a unique eosinophil constituent (Olsson *et al.*, 1977). The normal density found for eosinophils of patients with parasite infections, eosinophil gastroenteritis, primary hepatocellular carcinoma and lymphoma suggest that eosinophil degranulation in the circulation is not a significant phenomenon in these disorders.

Our findings in HES of a mixture of eosinophils populations suggest activation by unknown mechanisms of some eosinophils. Light eosinophils of HES may represent a subpopulation of 'activated' eosinophils with a prolonged half-life in the circulation. Actually the blood half-life of Indium-labelled eosinophils was sometimes prolonged in hypereosinophilia (Spry, 1980). An activating process might explain for findings in such cells of increased expression of cell surface receptors, increased spontaneous oxygen consumption and an increased respiratory burst upon adherence to a complement-activated surface. It is at least unlikely that such changes reflect the eventual preleukaemic nature of eosinophils of HES even if leukaemic eosinophils were found to have an abnormal low density. Our finding of exudate eosinophils with lower density and higher spontaneous oxygen consumption than blood eosinophils suggests that eosinophils may be activated normally in the tissues to change their properties. In HES this process may occur prematurely in the circulation for unknown reasons and explain the presence of

'activated' eosinophils of low density. It is of interest that in one of our patients with HES, a febrile episode resulted in a profound decrease of 'heavy' eosinophils while 'light' density eosinophils actually increased. This process may represent activation in the circulation of eosinophils with a concomitant shift in cell density.

In a recent report eosinophils from patients with eosinophilia appeared activated when compared with normal eosinophils by surface charge, activation of acid phosphatase and hexose transport (Bass *et al.*, 1980). Interestingly, activation of oxidative metabolism was found separable from activation of cell charge or hexose transport. Both high spontaneous and/or stimulated respiration as found in the present study of some light density eosinophils as well as degranulation could result in cytotoxic damage to tissues in HES because of active oxygen products, and released ECP and MBP.

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BIOCHEMICAL PROPERTIES OF THE EOSINOPHIL CATIONIC PROTEIN (ECP) AND STUDIES OF
ITS BIOSYNTHESIS IN VITRO IN MARROW CELLS FROM PATIENTS WITH EOSINOPHILIA

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ABSTRACT

The eosinophil cationic protein (ECP), which has been shown to be secreted both in vitro and in vivo is a cytotoxic unique constituent of eosinophil granules. To increase the understanding of the mechanisms behind the role of the eosinophil as a cytotoxic effector in disease a detailed characterization of ECP was performed. A considerable molecular heterogeneity was revealed when purified ECP was eluted isocratically from a high resolution cation exchange resin; the separation reproducibly achieved of 5 components was probably due to hydrophobic interaction with the resin. These components reacted quantitatively with anti-ECP antiserum, showed molecular weights of 19,500 and 16,700 and showed almost identical amino acid compositions. The amino-terminal sequence for one of these components was (in the standard one-letter code) (R-P-X-Z-F-T-R-A-Z-W-F-A-I-Z-H-I-S-L-N-P-R-R-C-T-I-A-M-R-A-I-N-N-Y-). The biosynthesis of ECP was demonstrated in marrow cells from patients with eosinophilia using labeling with (^{14}C)-leucine followed by immunoprecipitation with anti-ECP, sodium-dodecyl sulphate polyacrylamide gel electrophoresis and fluorography for visualization of labeled ECP. Biosynthesis was demonstrated of M_r 22,000 ECP, which may represent precursor ECP since with time some of it was processed into M_r 18,000 ECP. Monensin, a proton ionophore blocked the processing of M_r 22,000 ECP.

INTRODUCTION

The human eosinophil granule is characterized by its rich content of highly cationic proteins. Among these are the major basic protein (MBP) (1, 2), the eosinophil cationic protein (ECP) (3, 4) and the eosinophil-derived neurotoxin (EDN) (5). Both MBP, ECP and EDN represent distinctive cationic proteins of eosinophils as judged by their immunochemical and physicochemical properties (6). After secretion these strongly basic proteins are likely to be responsible at least in part for the cytotoxic effects of eosinophils on parasites (7, 8) and cells (9, 10, 11) although the specific effects have not yet been defined. Detailed characterization of the eosinophil cationic agents should therefore lead to an understanding of the mechanisms behind their roles as cytotoxic effectors and perhaps to an understanding of the functions of the eosinophil.

ECP represents a family of immunologically identical extremely basic molecules (3, 4) with an isoelectric point higher than pH 11. The major ECP constituent was reported to have a molecular mass of 21,000 daltons (3, 4), to contain 21 residues of arginine and to be a zinc containing protein. ECP does not show bactericidal or esterolytic activities (4) but damages schistosomula of *S. mansoni* (8) as well as cultured heart muscle cells (11). Furthermore it augments factor XII-dependent coagulation pathways (12), which may be of importance to explain thromboembolic complications of hypereosinophilia (13). No enzyme activity has as yet been reported for ECP.

In this work an extended purification and characterization of ECP was attempted by use of high performance ion exchange chromatography and the resolution was achieved of homogeneous polypeptides suitable for amino acid sequence analyses. Mechanisms behind the biosynthesis, processing, storage and secretion of granule proteins are largely unknown. Actually by use of monoclonal antibodies it was shown that one antibody recognized both storage and secreted forms of ECP whereas another antibody only bound to ECP during secretion (14). Thus solubilization of ECP in granules is associated with alterations in antigenicity. In the present work we therefore also tried to radio-label ECP biosynthetically by the use of bone marrow cells from patients with eosinophilia. Such cells were incubated with (^{14}C)-leucine and ECP was isolated by immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography for visualization of newly synthesized ECP. The results indicated that ECP is subjected to alterations in size subsequent to biosynthesis.

MATERIALS AND METHODS

Materials. Sephadex G-75 superfine, CNBr-activated Sepharose 4B, Protein A-Sepharose CL 4-B and Mono S cation exchange columns were from Pharmacia, Fine Chemicals (Uppsala, Sweden). The Pharmacia FPLC system was used for chromatography. Acrylamid/Bis 29:1 was obtained from Bio-Rad Laboratories (Richmond, CA). L-(^{14}C)-leucine (342 mCi/mmol) and En³hance were from New England Nuclear. Phenylmethylsulfonylfluoride (PMSF) and Monensin were from Sigma Chemical Co (St. Louis, MO). Diaflo membranes were from Amicon (Lexington, Mass.).

Purification of ECP

ECP was isolated by methods previously described (3, 4). Briefly, leukocytes from patients with chronic myeloid leukemia (CML) with some eosinophilia and leukocytes from a patient with bronchial carcinoma with high eosinophilia were homogenized in 0.34 M sucrose followed by differential centrifugation to obtain a crude granule fraction. Granules were extracted with 10 volumes of 0.2 M sodium acetate buffer, pH 4. After concentration by ultrafiltration on a Diaflo PM-10 membrane, chromatography was performed on a Sephadex G-75 column and the material of the ECP-containing peak was further separated by ion exchange chromatography on E-aminocaproic acid Sepharose. These procedures resulted in the isolation of a family of essentially pure ECPs reacting with an antibody against the major ECP component, which had been purified by electrophoresis on agarose (3, 4). The antibody was produced by immunization of rabbits with the homogenous major ECP component.

The isolated ECPs were analyzed by chromatography on a 1 ml Mono S cationic exchange column using the Pharmacia fast pressure liquid chromatography (FPLC) system. Elution was with a gradient of sodium acetate pH 5.0 at a flow rate of 1 ml per min.

Amino acid composition was determined by using an automatic amino acid analyser after hydrolysis in 6 M-HCl under argon at 110°C for 24 h.

Amino acid sequence analysis. Purified ECP was reduced and carboxymethylated with iodo(^{14}C)acetic acid in 6M guanidine-HCl. Automated amino acid sequence analysis by the method of Edman and Begg (15) was carried out on a Beckman 890C sequencer by use of the standard Beckman 1M Quadrol program. All fragments were run in the presence of Polybrene (16). Phenylthiohydantoin (PTH) derivatives of

amino acids were identified by high-performance liquid chromatography (HPLC) on a 30-cm Waters u-Bondapak C18 column using stepwise elution with methanol-containing buffers for the ethylacetate phases and acetonitrile-containing buffers for the aqueous phases. PTH derivatives of amino acids were also identified by thin-layer chromatography (17). PTH-carboxymethylcysteine residues were also identified by measurements of radioactivity.

Isolation of marrow cells. Bone marrow cells from five patients with eosinophilia were collected in heparinized Mc Coy's medium and erythrocytes removed by dextran sedimentation. The cells from the supernatant were washed and used for biosynthetic labeling. Four patients had an idiopathic hypereosinophilic syndrome with blood eosinophils in the range of 3.0 to $14 \times 10^9/L$ and a duration of the illness from 1 to 13 years. One patient had eosinophilia secondary to a malignant lymphoma.

Labeling of cells. For biosynthetic labeling of ECP, marrow cells were starved in leucine-free MEM (Eagle) with 1% fetal bovine serum (FBS) for 60 min at 37° . The labeling medium was the same deficient medium with 5% FBS. The cells, $3 \times 10^6/ml$, were incubated with $15 \mu Ci/ml$ of (^{14}C)-leucine for 1-18 h. In chase experiments, cells were first pulsed with $50 \mu Ci/ml$ (^{14}C)-leucine for 60 min, washed and suspended in RPMI 1640 medium with 10% FBS with 10^6 cells/ml followed by incubation for various chase time periods.

Extraction of cells. A radioimmuno precipitation assay (RIPA) buffer consisting of 0.15 M NaCl, 30 mM Hepes pH 7.3, 1% (v/v) TRITON X-100, 1% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulfate (SDS) was used for extraction. Approximately 1 ml of RIPA buffer with 1 mM PMSF was added per 10^7 cells and the extracts kept in ice for 1 h followed by centrifugation at 4° at $32,000 \times g$ for 2 h. The clear supernatant was stored frozen until used for immunoprecipitation.

Immunoprecipitation of radioactive ECP. RIPA-buffer extracts, 100-300 μl were mixed with 15 μl anti-ECP. After standing at 4° 12-18 h, 40 μl of a Protein A-Sepharose (200 mg/ml) solution in RIPA-buffer was added for collection of the immunoprecipitate by rotation at 4° for 60 min. The protein A-Sepharose was washed 5 times with RIPA buffer and the supernatant was carefully removed. The pellet was suspended in 50 μl H_2O plus 15 μl electrophoresis sample buffer (0.4M TRIS pH 6.8, 50% glycerol, 10% SDS and 5% β -merkaptoethanol), boiled for 5 min and used for electrophoresis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Fluorography

SDS-PAGE was performed on slab gels 18 cm long, 1.5 mm thick, and 16 cm wide, with 10 wells, in a LKB 2001 Vertical Electrophoresis unit (LKB Products, Bromma, Sweden) according to Laemmli (18). Samples were loaded on a linear gradient of 5 to 20 % polyacrylamide gel with 3% stacking gel. The electrophoresis was run at 25 mA/gel without cooling. After electrophoresis, gels were fixed in 10% TCA, 10% acetic acid, and 30% methanol for at least 1 hr, treated with Enhance for 1 h and H_2O for 1 h. Gels were dried on filter paper, and exposed to X-ray film (Kodak X-Omat S) at -80° for 3-6 days.

Apparent molecular weights were determined by use of the following (^{14}C) methylated standards (New England Nuclear): cytochrome C, 12,300; carbonic anhydrase, 30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; and phosphor-ylase B, 97,400.

To quantitate radioactivity, individual bands, localized on the dried gel using fluorography, were excised and treated overnight at $37^{\circ}C$ in 1 ml of a solution containing 95 parts 30% H_2O_2 and 5 parts 30% NH_3 . Fifteen ml of scintillation liquid was added for counting.

RESULTS

High resolution ion exchange chromatography of ECP

ECP isolated by gel filtration followed by E-aminocaproic acid Sepharose chromatography from blood eosinophils of a patient with bronchial carcinoma and high eosinophilia showed 2 distinct components when analyzed by SDS-PAGE. The molecular weights of these components were 19,500 and 16,700. All ECP was eluted from a Mono S column with approximately 1.0 M sodium acetate pH 5.0 without achieving separation between ECP components. Surprisingly, however, a considerable heterogeneity was revealed by isocratic elution with 0.9 M sodium acetate, which resulted in the resolution of 5 separate components (Fig. 1). As demonstrated in Fig. 1 all components reacted quantitatively in a radial immunodiffusion assay using an antiserum raised against one homogeneous ECP species isolated by preparative electrophoresis (3, 4). Thus a marked microheterogeneity for ECP molecules is found. ECP of the first two peaks eluted from the Mono S column had a molecular weight of 19,500 while that of the three peaks eluted later had a molecular weight of 16,700 (Fig. 1). The elution pattern of ECP was highly reproducible and an identical separation pattern as

in Fig. 1 occurred in another experiment with ECP purified from eosinophils of a patient with the idiopathic hypereosinophilic syndrome (data not shown). This result rules out that the elution profile of ECP on Mono S separation is an artefact.

For detailed biochemical characterization of ECP abundant material isolated from eosinophils from patients with chronic myeloid leukemia was utilized. This ECP was obtained from the Mono S column in 4 peaks by isocratic elution with 0.9 M sodium acetate pH 5.0 (Fig. 2). By rechromatography twice of peak B a homogeneous polypeptide species was obtained with a molecular weight of 18,000 (Fig. 3). Rechromatography once of peak D resulted in a homogeneous polypeptide with a molecular weight of 16,700 (Fig. 3). ECP of peak C was difficult to isolate in a homogenous state by rechromatography.

Amino acid analyses

Table I shows amino acid analyses of ECP components A-D isolated by high resolution ion exchange chromatography (Figs 2-3). The amino acid compositions are almost identical for all ECP components.

Amino acid sequence of ECP

The amino acid sequence of the NH_2 -terminus is shown in Fig. 4. Thirtythree amino acid residues were sequenced. The third residue could not be identified.

Biosynthesis of ECP

Marrow cells from a patient with CML and a patient with eosinophilia were incubated with (^{14}C)-leucine for different times followed by extraction and immunoprecipitation with anti-ECP and SDS-PAGE. The fluorograms (Fig. 5) show that eosinophilia marrow cells synthesized ECP polypeptides with a molecular weight of 22,000. With time CML marrow cells produced polypeptides with molecular weights of 25,000 and 18,000. Eosinophil marrow cells were also pulsed with (^{14}C)leucine for 1 hr and the label was chased for 1, 3, 7 and 18 h and the fluorograms obtained after SDS-PAGE are shown in Fig. 5. The pulse labeling experiments show that some of the newly synthesized M_r 22,000 ECP polypeptide was with time converted into ECP with a molecular weight of approximately 19,000. The presence of Monensin, a carboxylic proton ionophore that exchanges preferentially Na^+ ions for protons, prevented the conversion of M_r 22,000 ECP into lower molecular weight products.

The time course for incorporation of (^{14}C)-leucine into M_r 22,000 ECP is shown in Fig. 6. Individual bands corresponding to the M_r 22,000 polypeptide were localized on gels using fluorography, excised and counted. Incorporation increased for at least 15 hr.

Fig. 7 shows results from ECP biosynthesis studies of marrow cells in 5 patients with hypereosinophilia. In all the major ECP product has a molecular weight of 22,000 while a minor product has a molecular weight of approximately 18,000. Monensin prevented the formation of the M_r 18,000 ECP. Also total biosynthesis of ECP was inhibited by Monensin to a variable degree.

DISCUSSION

One approach to the understanding of eosinophil function is to study the physicochemical properties and biologic actions of eosinophil granule constituents. Eosinophil cationic polypeptides of granules may be responsible for cytotoxic effects of eosinophils on parasites and cells. In this work we show that an extended separation of purified ECP revealed a considerable molecular heterogeneity. Thus by isocratic elution from a high resolution cation exchange resin, five ECP components were resolved. The separation was probably not a result of ion exchange but more likely a result of partition chromatography due to hydrophobic interactions between ECP and the resin. Although the different components showed slight differences in molecular mass judging from SDS-PAGE, the amino acid compositions were almost identical.

The separation pattern on the cation exchange resin of ECP components was highly reproducible; ECP purified from different patients with eosinophilia showed an identical elution profile. Future work will show if the ECP components obtained differ in biologic activities.

The sequence determination of the amino-terminal part of one ECP component revealed a unique sequence. Thus when this sequence was aligned with known protein sequences in a computerized search, no specific homologies were found. Minor homologies with the receptor-binding site of diphtheria toxin (comprising 8 of the sequenced 33 aminoacids of ECP) are probably without significance.

In the present study ECP was purified from granule extracts. Eosinophils have an ability to secrete ECP both in vitro (19) and in vivo (20). It is not known, however, if all forms of ECP as detected in the present work are secreted to

the same extent. The secretory process has been investigated by the use of monoclonal antibodies against ECP (14). One antibody to ECP bound to eosinophils from both normal individuals and patients with eosinophilia. A second antibody, also recognized ECP but was specific for the secreted form of the protein. The results suggest that there are structural alterations of ECP during secretion.

In another approach to study structural modification of ECP, biosynthetic labeling was used followed by immunoprecipitation with anti-ECP. Marrow cell specimens from patients with eosinophilia were used for these experiments. Such specimens contain a mixture of eosinophils at all stages of maturation. However, relevant biosynthesis and intracellular transport of ECP occur only in immature eosinophils at the stage of maturation where granules are formed. All experiments demonstrated biosynthesis of ECP with a molecular weight of 22,000. This form does not correspond to any of the ECP forms purified from eosinophil granules and may therefore represent a precursor polypeptide for ECP, which is subject to processing during intracellular transport and storage in eosinophil granules. Consistent with this interpretation some M_r 18,000 ECP appeared with time. This was most clearly shown in pulse-chase labeling experiments. The processing of M_r 22,000 ECP into M_r 18,000 ECP was, however, not a very efficient event, which may indicate that the cellular system was not optimal enough or that ECP processing is very slow.

Monensin, a proton ionophore, blocked the processing of newly synthesized ECP and also inhibited to a variable degree the biosynthesis of ECP. This is explained by the known inhibitory effects of Monensin on transport of secretory or lysosomal proteins and related to Monensin's ability to raise the normally acidic pH of the vacuolar system of the cell.

Additional structural studies of ECP will require the use of DNA hybridization probes complementary to ECP-mRNA. Knowledge of the amino-terminal sequence of ECP can be used for synthesis of corresponding oligonucleotides to be used as probes for screening of a cDNA-library established from marrow cells of patients with eosinophilia.

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TABLE I

Amino acid composition of ECP components A-D separated by high resolution ion exchange chromatography (Figs 2-3). Data are given as residues/100.

	A	B	C	D
Aspartic acid	16.4	16.6	16.4	15.4
Glutamic acid	7.4	5.9	6.7	5.8
Glycine	6.2	4.4	5.1	5.0
Alanine	7.7	6.3	7.1	7.1
Valine	8.0	7.4	8.2	8.0
Leucine	5.2	6.0	6.4	6.5
Isoleucine	5.3	6.2	6.2	6.2
Serine	6.2	4.5	4.8	4.3
Threonine	6.0	5.8	5.8	5.6
Methionine	-	-	-	-
Proline	9.8	9.3	8.8	11.6
Phenylalanine	3.1	4.6	4.4	4.3
Tyrosine	0.6	1.7	0	2.0
Histidine	4.0	4.2	4.3	4.1
Lysine	1.1	1.1	1.0	0.9
Arginine	12.9	14.8	14.8	14.9

Cysteine and Tryptophan were not quantitatively determined.

Methionine was barely detectable but the amino acid sequencing of the NH_2 -terminus of component D revealed one methionine residue.

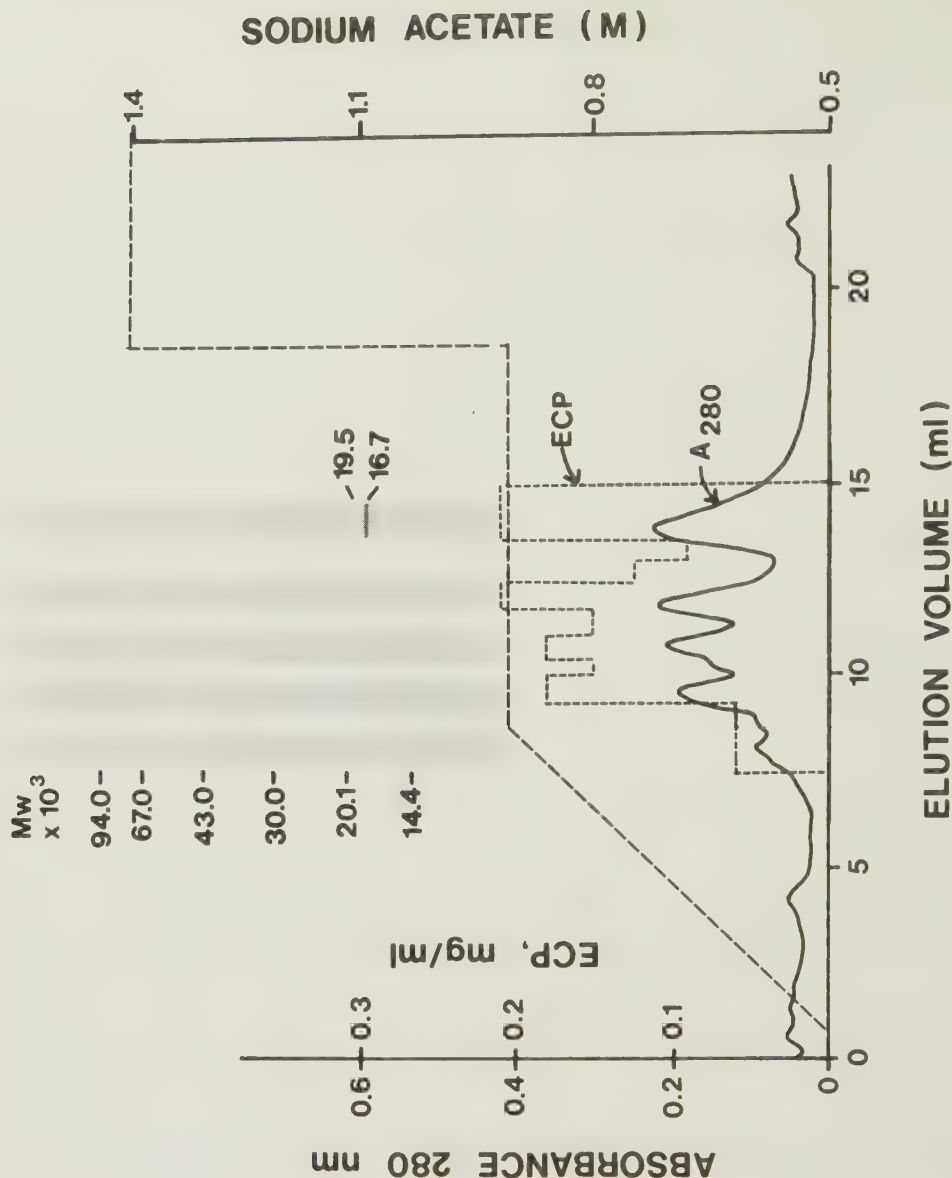


Fig. 1. High resolution chromatography of ECP. ECP, 1 mg, isolated from blood eosinophils of a patient with bronchial carcinoma using gel chromatography and E-aminocaproic acid Sepharose chromatography, was subjected to high resolution chromatography on Mono S using the FPLC system. Elution was with sodium acetate pH 5.0 (----) at a flow rate of 1 ml/min. The absorbance at 280 nm is monitored as well as the ECP content of fractions determined by radial immunodiffusion (-----). Included is also the SDS-PAGE pattern of ECP eluted in various fractions; molecular weight markers are to the left.

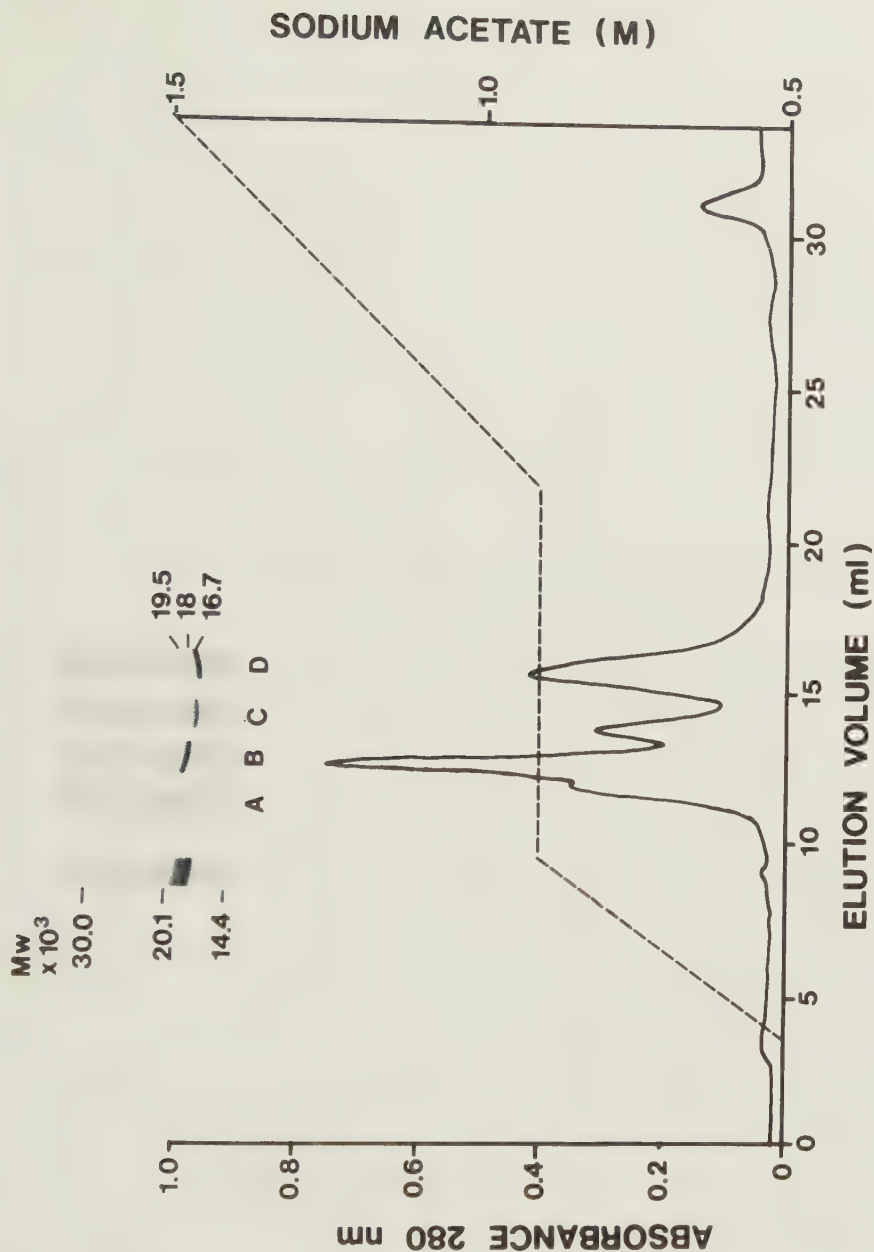


Fig. 2. High resolution chromatography of ECP. ECP, 2.5 mg, isolated from blood leukocytes of a patient with CML using gel chromatography and E-aminocaproic acid Sepharose chromatography. Elution was with sodium acetate pH 5.0 (----) at a flow rate of 1 ml/min. The absorbance at 280 nm is monitored. The SDS-PAGE pattern of ECP eluted in different peaks (A-D) is included as well as the SDS-PAGE pattern of the ECP lot used for the Mono S chromatography (far left); molecular weight markers to the left.

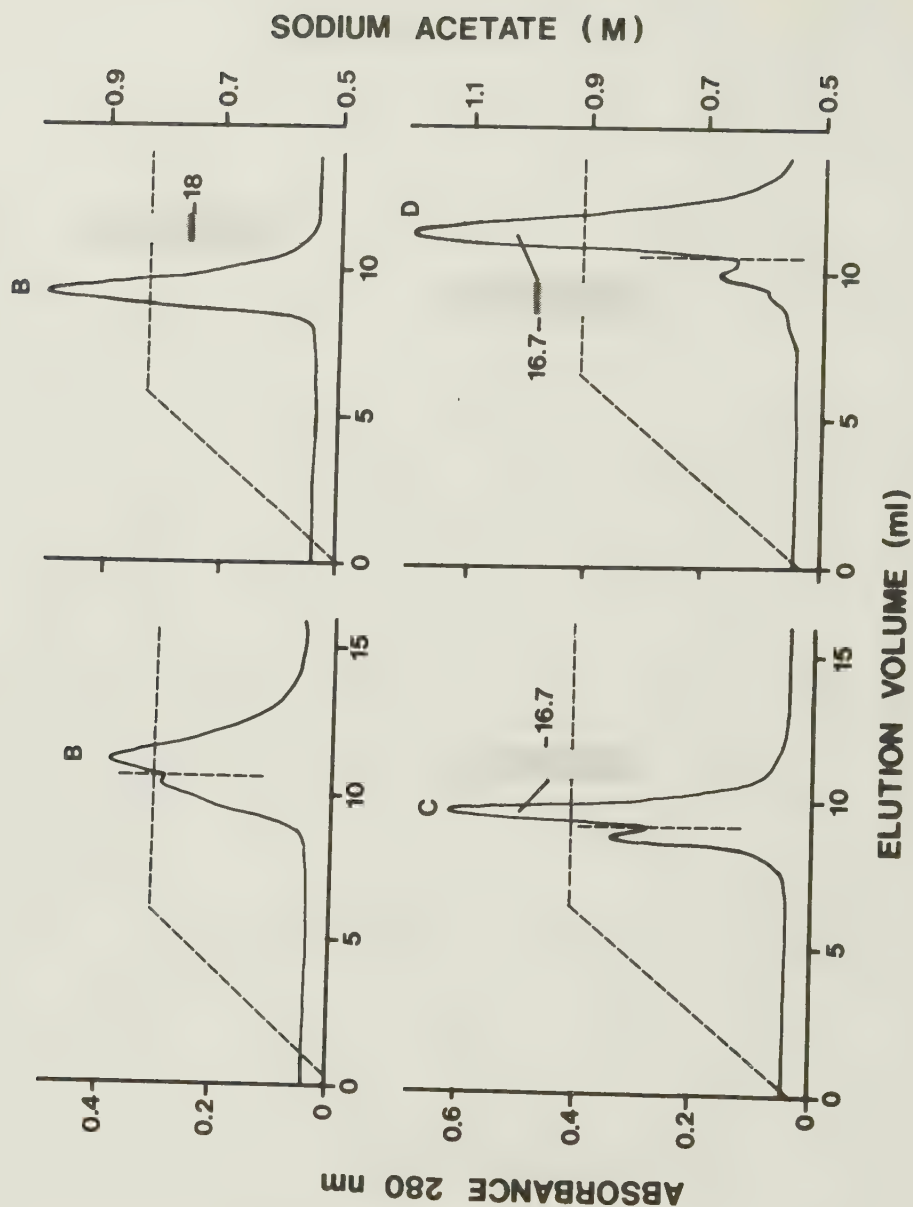


Fig. 3. High resolution chromatography of ECP. Rechromatography on Mono S of ECP components B, C and D of the chromatogram shown in Fig. 2. Elution was with sodium acetate pH 5.0 (----) at a flow rate of 1 ml/min. The absorbance at 280 nm is monitored. Component B was rechromatographed twice (upper part of figure). The SDS-PAGE pattern is included. As shown components B and D were purified to homogeneity and component D was used for amino acid sequence analysis.

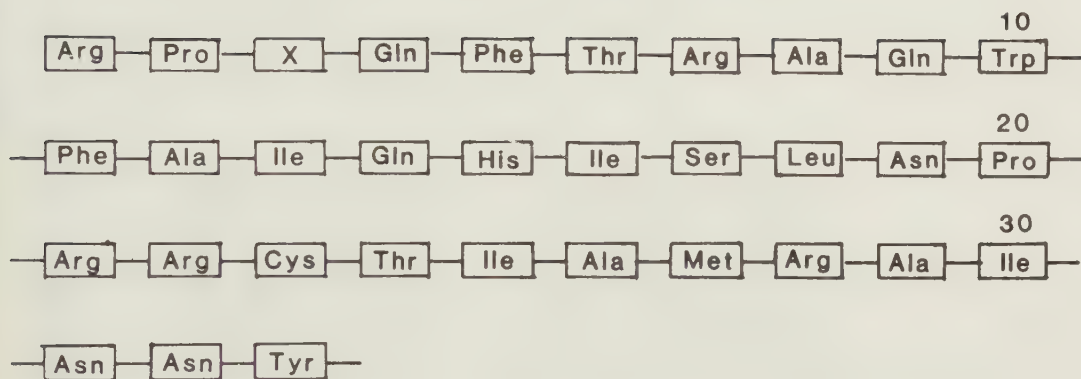


Fig. 4. The NH₂-terminus amino acid sequence of ECP (component D of Fig. 3).

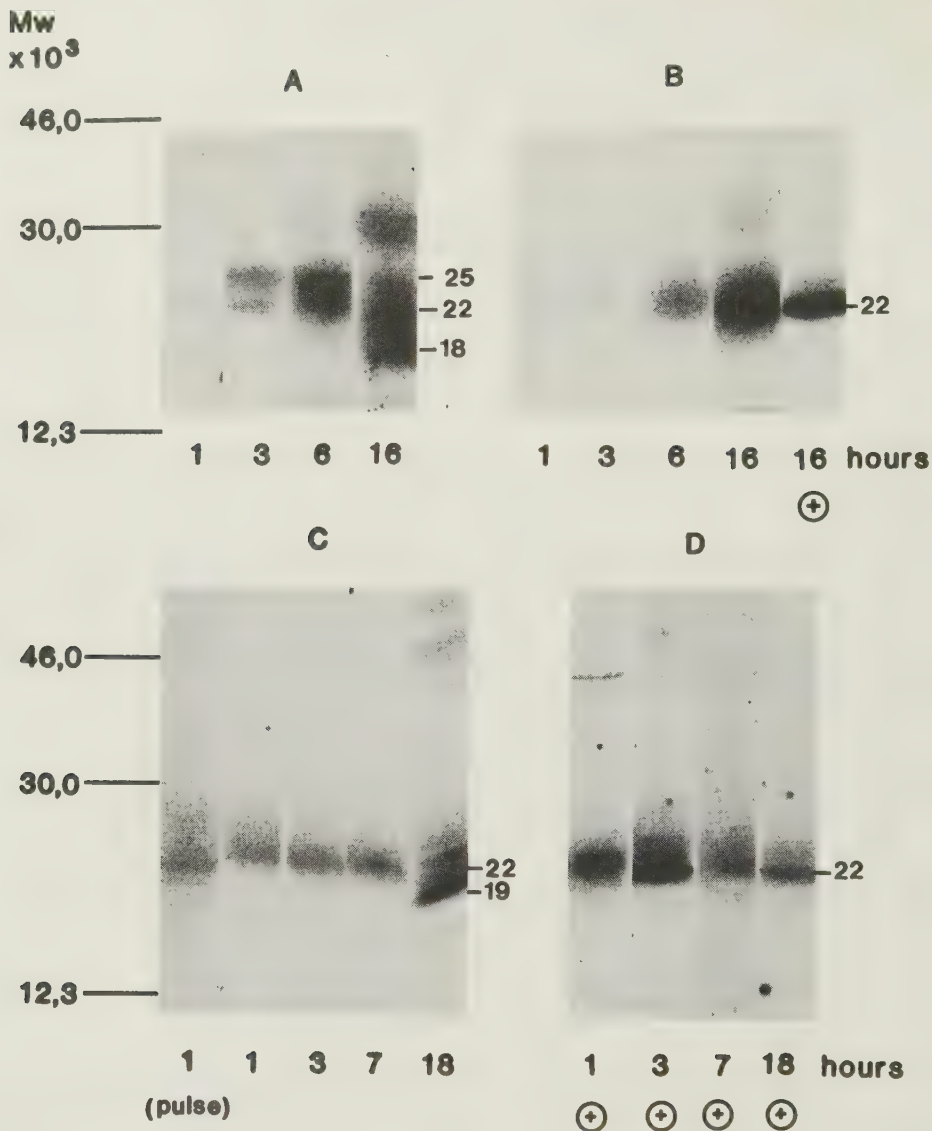


Fig. 5. Biosynthetic labeling of ECP. Marrow cells from a patient with CML(A) or eosinophilia (B, C and D) were used. In A and B cells were incubated for various times with (¹⁴C)-leucine followed by extraction, immunoprecipitation with anti-ECP and SDS-PAGE. In C and D cells were pulse-labeled with (¹⁴C)-leucine 1 hr and the label was chased for up to 16 hr. Some incubations were performed in the presence of 1 μM Monensin (+). Molecular weight markers are shown to the left; molecular weights of major ECP components are also shown.

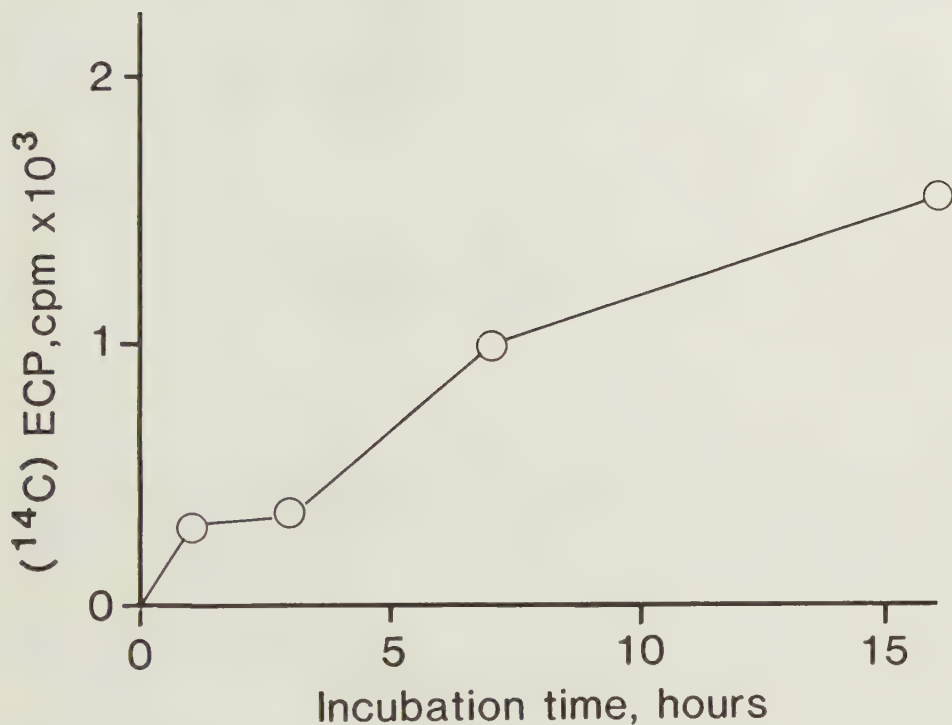


Fig. 6. Time course for incorporation of (^{14}C) -leucine into ECP. Cells were incubated for various times with (^{14}C) -leucin (corresponding to B in Fig. 4) followed by extraction, immunoprecipitation with anti-ECP and SDS-PAGE. Individual bands corresponding to the M_r 22,000 polypeptide were localized on the dried gel using fluorography, excised and counted by liquid scintillation.

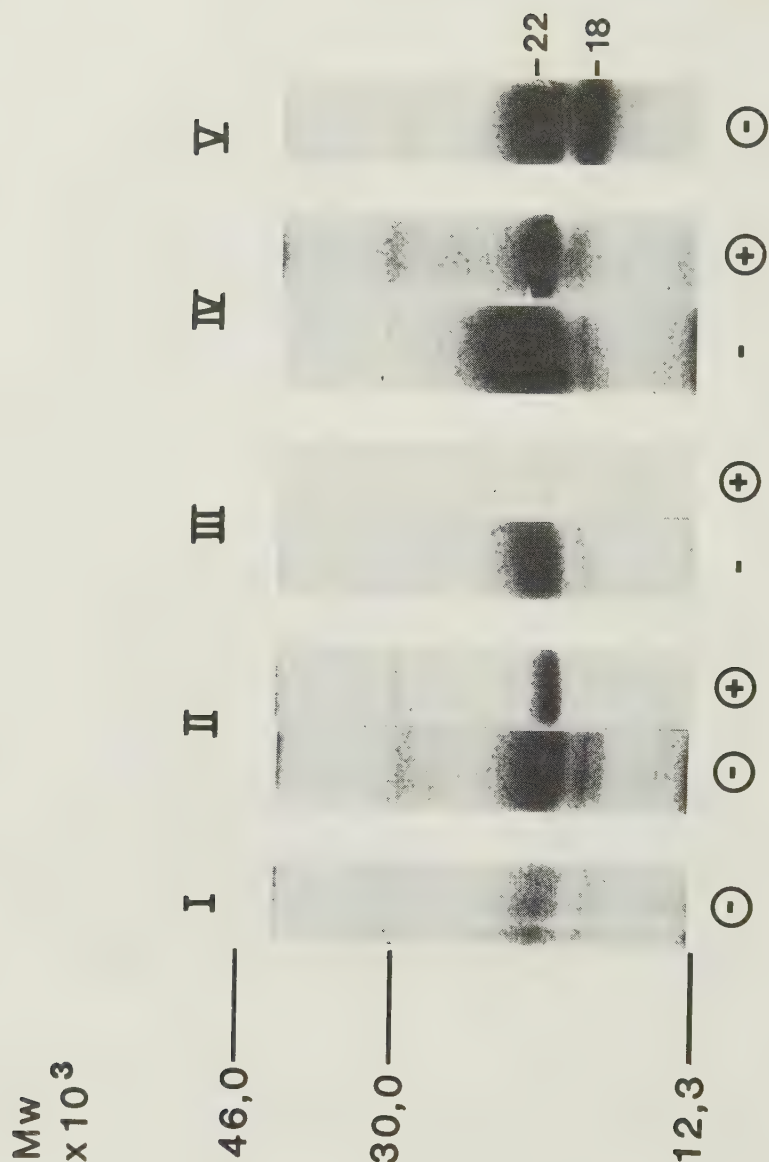


Fig. 7. Biosynthetic labeling of ECP. Marrow cells from 5 patients (I-V) with hypereosinophilia were used. Cells were incubated for 16 hr with (^{14}C)-leucine followed by extraction, immunoprecipitation with anti-ECP and SDS-PAGE. Cells were incubated without (-) or with (+) 1 μM Monensin. Molecular weight markers are shown to the left; molecular weights are also indicated for labeled major ECP components.





